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DNA-DNA Hybridization Phylogeny of Sand Dollars and Highly Reproducible Extent of Hybridization Values

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Summary. A DNA hybridization phylogeny of four sand dollars using a sea biscuit as an outgroup is presented. The study is unusual in that the normalized percent hybridization (NPH) values were all <50%, yet the same topology was obtained regardless of which distance metric was used, i.e., whether reciprocal distances were averaged or not, or whether or not a molecular clock was assumed. The tree also appears robust under jackknifing and bootstrapping. The extent of hybridization between homologous hybrids was measured with a five- to sevenfold higher precision than is typical, and by implication NPH was also measured with a higher than normal precision. The ability to measure highly reproducible NPH values offers the possibility of examining the phylogeny of more widely divergent species than typically studied using DNA hybridization techniques, using 1/NPH as a distance metric. The hypothesis of a molecular clock within the sand dollars was rejected, adding sand dollars to the growing list of groups where significant rate variation is known. A small fraction of the sand dollar genomes hybridized with the distantly related regular sea urchin *Lytechinus*. These slowly evolving sequences probably represent conserved exonic components of the genome.

Key words: DNA/DNA hybridization — Phylogeny — Clypeasteroids — Sand dollars — Normalized percent hybridization — Genome evolution

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Introduction

DNA hybridization techniques have been used to explore evolutionary relationships for more than 20 years (Hoyer et al. 1964; Britten and Kohne 1968), though it is only in the last decade, and largely due to Sibley and Ahlquist's extensive work on birds and hominoids, that the technique has gained a wide audience. With wider attention, the use of the technique for phylogenetic purposes has come under close methodological scrutiny. The areas attracting most interest have been: the overall usefulness of DNA hybridization data for phylogenetic reconstruction (Bledsoe and Sheldon 1989, 1990; Sarich et al. 1989; Springer and Krajewski 1989a; Diamond 1990), especially within the Hominoidea (Sibley and Ahlquist 1984, 1987; Felsenstein 1987; Marks et al. 1988, 1989; Britten 1989; Caccone and Powell 1989; Sibley et al. 1990), and birds (Lanyon 1985; Cracraft 1987; Sibley et al. 1987). Also discussed has been the most appropriate metric for measuring phylogenetic distances (Sarich et al. 1989; Sheldon and Bledsoe 1989; Britten 1990), the role of the molecular clock in constructing phylogenies (Bledsoe 1987a,b; Houde 1987a,b; Sheldon 1987; Springer and Krajewski 1989b), and the best experimental procedures for deducing phylogenies (Hall et al. 1980; Powell and Caccone 1990; Schmid and Marks 1990). For an excellent review of the different techniques see Werman et al. (1990a).

The major aim of this paper is to report a DNA hybridization phylogeny of sand dollars. Despite the fact that all interspecies normalized percent hybridization (NPH) values were less than 50% the phylogeny derived from the hybridization data appears

robust. This phylogeny differs from the majority of morphologically based phylogenies (Seilacher 1979; Jensen 1981; Smith 1984; Mooi 1987). Reconciliation of the conflicting morphological and DNA hybridization data is discussed at length elsewhere (Marshall 1992). A second aim is to report a higher than usual reproducibility in the measurement of the extent of hybridization for homologous hybrids. With careful attention to experimental procedure it may be possible to examine the relationships between species with more widely divergent DNAs than usually undertaken with DNA hybridization techniques.

Our data indicate that if DNA reassociation is measured with high precision a particularly robust phylogeny can result. Although it may sometimes be necessary to study DNA reassociation kinetics from DNA obtained under less than optimal field conditions, when accurate determinations are to be obtained it is desirable to isolate DNA under controlled conditions. This is particularly true for the determination of small differences between closely related species, or conversely, as here, between widely divergent genomes where the degree of hybridization is relatively small.

Our study also has implications for the rates of sequence change in evolution. Not only are the rates of change markedly different when interspecies hybridizations are compared between species pairs, but also different sequences within the genome change at vastly different rates. Comparisons of reassociations between divergent genomes widely spaced in time (as is the case reported here) indicate the presence of a fraction of sequences whose rates of change are very low, probably representing in part the conservative housekeeping components of the genome.

Sand dollars were chosen for this study, not only because their complex morphology provided the opportunity for a rigorous comparison of the DNA hybridization phylogeny reported here with the existing morphological phylogenies, but also because the group has a tremendous fossil record (Marshall 1988). Marshall (1989) provides a detailed examination of the fossil record of the group in an effort to assess the rate of single-copy DNA evolution in the group. The phylogenetic study involves four scutelline sand dollars: the dendrasterid *Dendraster excentricus* (Eschscholtz) (*D.e.*), the echinarachniid *Echinarachnius parma* (Lamarck) (*E.p.*), and the mellitids *Leodia sexiesperforata* (Leske) (*L.s.*), and *Mellita* spp. (*M.t.*). The clypeasterine *Clypeaster rosaceus* (Linne) (*C.r.*) was used as an outgroup. The hybridization techniques employed were essentially those of Benveniste and Todaro (1974, 1976), Benveniste et al. (1977), and Benveniste (1985); S1 nuclease rather than fractionation on hydroxyapatite was used to measure the percent reassociations and melting temperatures, and the hybridizations and melting of hybrids were carried out in NaCl solutions.

Materials and Methods

DNA Hybridizations

Tissue Collection. Spawning was induced in the species studied (Table 1) by injecting animals through the peristomial membrane with 0.5–2 ml 0.55 M KCl. Sperm were collected and kept on ice until frozen (within an hour) in liquid nitrogen or on dry ice.

Mellita. The *Mellita* from Gulf Specimen Inc. (Panacea, FL) were presumed to be the newly recognized *Mellita tenuis* Harold and Telford 1990. However, of two specimens sent for identification one was determined to be *Mellita isometra* Harold and Telford 1990 (Telford, personal communication), thought only to occur on the Atlantic coast of Florida. At the time of collection both species were classified as *Mellita quinquiesperforata,* and no attention was paid to the small differences between individuals; thus, the sperm collected may have come from a mixed group of *Mellita.* However, there was no depression in the homologous T_m of *Mellita* over the T_m's of the other clypeasteroids, which implies that either the sperm was derived largely from just one of the two species, or *M. tenuis* and *M. isometra* are so closely related that the interspecific distance between the species is no larger than the average intraspecific distance seen between the other sand dollars analyzed. At present it is impossible to distinguish between these two possibilities.

Extraction and Purification of DNAs. DNA was extracted from frozen sperm using the modified Mamur method (Britten et al. 1974; Table 2), with the following modifications: twice the recommended volumes of SEDTA [0.1 M NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and lysing mixture (SEDTA, 1% SDS, and 0.2 mg proteinase K) were used to facilitate the extractions, and the recovered DNA was treated with 0.05–0.1 mg/ml RNAase A for 3–5 h at 37°C, followed by extraction with one volume of phenol and one volume of IAC before reprecipitation and final recovery either by spooling or centrifugation.

DNA purity was tested spectrophotometrically and was deemed clean if the absorption ratios A260/A280 ≥ 1.8 and A260/A230 ≥ 2.3. Hyperchromicities $[(A260_{100°C} - A260_{22°C})/(A260_{22°C})]$ were also measured and lay between 32 and 38%. The DNAs were run out on 0.8% agarose minigels to test for impurities and degradation and always formed well-defined bands approximately 22–25 kb in length.

Shearing and Sizing Driver DNAs. DNAs were sheared using a Branson S75 sonifier. After shearing, NaAc, pH 7.6, was added to a concentration of 0.3 M and the solution was spun in a desktop centrifuge at 1700 × *g* for 5 min to bring down the metal chips shed by the sonifier tip. The solution was transferred to another tube and the DNA was precipitated with two volumes of ethanol and resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8) to at least 3 mg/ml. The size range of the single-stranded sheared DNAs was determined on 1.5% alkaline agarose minigels run against a 123-bp ladder (Bethesda Research Laboratories), and DNAs ranged in size from 100 to 1100 bp, with a median size of approximately 400–500 bp.

Preparation of Tracer DNAs. Tracer preparation largely followed the procedure of Cunningham et al (1991). In isolating the single-copy DNAs it was noted that for a taxonomically diverse

Table 1. Sea urchin species included in study

Species	Locality collected	No. of males contributing sperm	Genome size (haploid, pg/cell)[a]
Dendraster excentricus (*D.e.*)	Southern California[b]	22	1.1
Echinarachnius parma (*E.p.*)	Woods Hole, MA[c]	25–35	1.1
Leodia sexiesperforata (*L.s.*)	Discovery Bay, Jamaica;	13	?
	Fort Pierce, FL	7	
Mellita tenuis (*M.t.*)	Gulf coast, FL[d]	19	0.98
Clypeaster rosaceus (*C.r.*)	Discovery Bay, Jamaica	1	?
Lytechinus variegatus (*L.v.*)	Discovery Bay, Jamaica	6	0.97

[a] Data from Hinegardner (1974)
[b] Supplied by Pacific Bio-Marine Laboratories, Inc., Venice Beach, Los Angeles
[c] Supplied by Marine Biological Laboratory
[d] Supplied by Gulf Specimen Company, Inc., Panacea, FL

range of clypeasteroids, including the clypeasterine *Clypeaster japonicus,* the laganine *Peronella japonica,* and two scutellines *Astriclypeus manni* and *D. excentricus,* the single-copy DNA contents constitute from 45% to 56% ($Cot_{½}$'s from 505 to 825 nucleotide-mol s/l) of the total genome (Yanagisawa 1988). Given the uniformity of genome size (Hinegardner 1974; Table 1) and organization among the clypeasteroids (Yanagisawa 1988), the DNAs were incubated to the same equivalent Cot (approximately 700). Forty micrograms of genomic DNA, sheared to a median size of 800 bp, were denatured by boiling for 10 min and then incubated overnight to ECot 700 [see Britten et al. (1974) for definition and method of calculating ECot] in 50 μl 0.6 M NaCl at 60°C. DNAs were then run through 300 μl hydroxyapatite (HAP) in a 1-ml syringe, and the single-stranded DNA was eluted by passing 0.12 M phosphate buffer (PB) over the column. Geneclean (BIO 101) was used to concentrate the DNA and remove the PB.

Between 30 and 300 ng of single-copy DNA was labeled with tritiated thymidine [(^{3}H)TTP] using a Boehringer-Mannheim Random Primed DNA labeling kit. The single-copy DNA was not boiled before labeling but heated to 70°C so that any contaminating double-stranded DNA (presumably repetitive DNA) that did not bind to the HAP during isolation of the single-copy DNA would remain double stranded and thus not be labeled (Caccone and Powell 1989; Cunningham et al. 1991). Unincorporated label was removed by passing the DNA over a Sephadex G-50 column (Maniatis et al. 1982) pre-equilibrated with 0.12 M PB. Random priming may produce large quantities of snap-back DNA, and the labeled DNA was denatured by boiling for 10 min and then allowed to incubate at 60°C for an hour to allow the snap-back DNA to renature. The DNA was then run over a HAP column to remove the snap-back DNA, which constituted 15–55% of the labeled DNAs. Geneclean (BIO 101) was again used to concentrate the DNA and remove the PB. In the tracer preparations a small aliquot was boiled, allowed to hybridize overnight at 60°C, and digested with S1 nuclease to determine if any significant snap-back DNA remained. The digestion of the labeled DNA was 97–98% complete.

The random priming produced fragments with a median size of around 250 bp, about half the size desired. To achieve a larger median tracer size the labeled DNA was size fractionated over a 1-ml Sephacryl S-400 column (Cunningham et al. 1991). Labeled DNA (≤50 μl) was added to the column prewashed with TE. The aliquots that showed significant radioactivity and largest median size were pooled [DNAs were sized on 1.5% alkaline agarose minigels following the general procedure of Hunt et al. (1981)]. Typically, enough labeled DNA for two complete sets of hybridizations was recovered with a median size of approximately 380 bp when two labeling reactions were combined.

Hybridization Conditions. Tracers were prepared for each clypeasteroid species, and each tracer was hybridized twice with all five clypeasteroids and the regular urchin *Lytechinus variegatus.* All tracers were also hybridized with calf thymus DNA (Cooper Biomedical, 50 mg dissolved in 10 ml TE, dialyzed against TE, boiled for 15 min and stored at −20°C) as a control. Limited quantities of DNA from some species prevented the running of any further complete rounds of hybridizations. Hybridizations were carried out in 1.00 (one case) or 1.08 M NaCl plus TE and 0.05% SDS in 1.5-ml Eppendorf tubes with O-ringed screw-on caps (Sarstedt). A few drops of mineral oil were added to retard evaporation.

Hybridizations were run to Cot values of between 1485 and 2040. The equivalent Cot values, assuming a rate acceleration factor of 7.1 for a 1 M NaCl solution (Britten et al. 1974) were 10,000–13,700. If the rate acceleration factor is actually 25 for 1 M NaCl solutions (Angerer et al. 1976) then the equivalent Cots were all greater than 38,000. The hybridizations were carried out in 10 μl/10 μg of driver and were incubated for 137–187 h at 60°C. The ratio of driver (10 μg/data point) to tracer (<1 ng = 4000 cpm/tube for S1 treatment) was always in excess of 10,000; in each tube approximately 10^3 genome equivalents (haploid) of labeled single-copy DNA was hybridized with 10^7 genome equivalents (haploid) of unlabeled driver DNA.

Hybrid Melting Temperatures and Determination of Extent of Reassociation. DNAs were allowed to hybridize at 60°C, which is 30°C, rather than the usual 25°C, below the homologous T_m values, to increase the NPH values. However, the effective criterion of precision is unknown. A comparison of the results of hybridizations between *Strongylocentrotus purpuratus* and *Strongylocentrotus franciscanus* in 1 M NaCl (Angerer et al. 1976) and in 0.5 M PB (Hall et al. 1980) shows that incubation in 1 M NaCl raises the effective criterion considerably. In addition S1 nuclease also increases the criterion over HAP techniques (Hall et al. 1980). After Cot values of >1485 were reached, each hybrid mix was diluted 20-fold, NaCl was added to a final concentration of 1.6 M, and 200 μl were aliquoted out into a series of tubes.

To determine the extent of hybridization two tubes (one tube for the second round of hybridizations) were set aside to measure the input counts and two tubes were treated with S1 nuclease. The ratio of S1 to non-S1-treated tubes (adjusted for background as discussed below) gave the extent of hybridization. The normalized percent hybridization (NPH) values were calculated by dividing the extent of hybridization for the species pair of interest by the extent of hybridization of the homologous hybrid. The extent of reassociation for homologous hybrids was typically 64% of the input counts, and thus all NPH values were elevated over the nonnormalized values of extent of reassociation.

To determine the melting temperature of the hybrid DNAs, a series of tubes (five in the first round, six in the second) was set aside. Each tube was heated to a successively higher temperature in a water bath for 5–6 min, immediately placed on ice, and then treated with S1 nuclease. In the first round, tubes were heated in 5–6°C increments; in the second round, having determined where the melting temperatures lay, the tubes were heated in 2.5°C increments. In each case a tube was also heated to 100–101°C and treated with S1 nuclease. In constructing melting curves, the number of radioactive counts measured at 60°C (used to measure NPH) was set to 0% single-stranded, and the level of background counts (discussed below) set the upper value of 100% single stranded.

S1 Nuclease Digestions. The hybrid DNAs were treated with S1 nuclease for 90 min at 37°C. One milliliter of S1 solution [40 mM NaAc, pH 4.5, 230 mM NaCl (to give a final concentration of 450 mM), 0.3 mM $ZnSO_4$, 5 μg calf thymus DNA (shared to 350 bp), 5% glycerol, and sufficient S1 nuclease (BRL) to digest 99% of the single strands present] was added to the 200-μl aliquots of hybrid DNAs. Digestion was stopped with 0.25 ml 50% w/v trichloroacetic acid (TCA). The tubes were placed on ice for 20 min and then filtered with approximately 10–15 ml 5% ice-cold TCA and 10–15 ml ice-cold 95% ethanol using Millipore 0.45-μm glass filters (type HA). Filters were oven dried and counted for 10 min in 3.3 ml Omnifluor in a Packard 3255 liquid scintillation counter.

Corrections to Raw Data

Background Counts. Background counts were derived from four sources (not including the ambient radiation): (1) undigested single-stranded tracer DNA; (2) snap-back tracer DNA; (3) tracer/tracer hybrids (most likely contaminating repetitive sequences); and (4) conserved sequences between the sea urchin and calf genomes (histone, cytochrome genes, etc.). The first two sources of background were regarded as the true background.

The baseline for estimating the true background was the number of counts that remained after the tracer/calf hybrid was heated to 100+°C and treated with S1 nuclease ($BG_{a:calf}$). The final value for the true background counts for each species pair was calculated by multiplying $BG_{a:calf}$ by the ratio of the input counts of the hybrid of interest to the input counts for the tracer/calf hybrid. For each experiment, the true background, 0.95–1.6% of the input counts, was subtracted from all values before any other calculations were made. Without further experiments it is difficult to distinguish between the two sources that contribute to the true background, but assuming both sources contribute equally (and ignoring the ambient radiation), the S1 nuclease digested approximately 99% of the single-stranded DNA present in each case, as was desired.

The counts associated with the extent of reassociation between the tracer and calf thymus DNAs were due to all four sources of background listed above. Given that the background due to shared, conserved, urchin–calf sequences (source 4) was minimal (1000 conserved genes would only contribute 0.2% to the extent of reassociation), the difference in total background counts and true background counts gives the background due to contaminating tracer/tracer hybrids, and values for this source of background lay between 1.0 and 2.2% of the input counts. Hall et al. (1980) report a similar value [2% when assayed on HAP and 3% when assayed in tetraethylammonium chloride (TEACL) with S1 nuclease] for the extent of reannealing between tracer DNA of the regular sea urchin *S. purpuratus* when incubated with 10,000-fold excess calf thymus DNA (as in our experiments).

Correcting for Multiple Hits. ΔT_m's were corrected for multiple hits using the equation provided by Jukes and Cantor (1969). The conversion from ΔT_m to percent mismatch has been variously given as 0.7–2.0°C for each percent NPH (McCarthy and Farquhar 1972; Caccone et al. 1988; Werman et al. 1990a), though in the literature 1°C for each percent NPH is most commonly used and was the value used here.

Intraspecific Variation. Homologous T_m's measured for DNAs prepared from multiple individuals will be depressed over homologous T_m's measured for DNAs prepared from a single individual due to intraspecific polymorphism (Britten et al. 1978). Reductions in thermal stabilities from intraspecific polymorphism ranged from 2 to 4°C (±0.5°C) for species of *Strongylocentrotus* (Grula et al. 1982). For our data, all homologous T_m's were based on DNA derived from approximately 20 males, except for *Clypeaster* where DNA was extracted from just one male (Table 1). *Clypeaster* and *Leodia* have similar hypochromicities (38%) and thus should have similar T_m values (the input tracer lengths were equal). The difference in their respective melting temperatures (1°C) was taken as a measure of the intraspecific sequence variation in sand dollars, and a correction factor of 1°C was added to the homologous T_m's of the four sand dollars before they were used to estimate the ΔT_m values.

Length Corrections. DNA melting temperature is a function of fragment length. For closely related species making length corrections to T_m greatly increases the agreement between replicate experiments (Caccone and Powell 1987, 1989). However, for widely diverged species, difficulties in locating exact T_m values due to large radioactive counting errors (Fig. 1) render length corrections of less importance.

The relationship between T_m and fragment length (L) is given by:

$$T_m(\text{long duplex}) - T_m(\text{duplex of length L}) = B/L$$

B is determined experimentally and equals 498 in 20 mM Na^+, 442 in 70 mM Na^+, and 750 in 196 mM Na^+ (see Hall et al. 1980). The value of B at 455 mM Na^+ (the value in our experiments) was not determined, but assuming a large value of B (say 1000), that the lengths of the homologous hybrids are 55% of the input DNA sizes (Smith et al. 1982), and a range of input sizes between 360 and 400 bp, the corrections to the T_m's would only range from 4.6 to 5.1°C. The input tracer lengths were more tightly constrained, lying between 370 and 390 bp. Thus, at most, the length corrections are likely to change the relative homologous T_m's by 0.5°C, and more likely by <0.3°C. Furthermore, it seems likely that the homologous hybrid fragment lengths are similarly sized, not only because of the care taken when preparing the input DNAs, but also because the T_m values correlate well with hypochromicity (for the four sand dollars, $r^2 = 0.917$), i.e., intraspecific differences in AT/GC content appear to account for 92% of the variance in the homologous T_m's.

AT/GC Ratio. T_m is a function of the AT/GC ratio in aqueous solutions. The AT/GC values for the clypeasteroids have not been measured directly; however, given that the homologous melting temperatures (88.4–89.8°C) for the group only span 1.5°C, and that T_m increases at about 0.44°C for each percent in GC content, then the maximum difference in GC content between any species pair is probably relatively small, about 3.5%.

Phylogenetic Analysis

Distance Metrics Used. Phylogenetic trees were computed using both ΔT_m and 1/NPH (used as an approximation to $\Delta T_m R$, see below) values. For distantly related species T_{mode} values were essentially impossible to estimate, due to the smearing typical of melting curves of hybrids between widely divergent DNAs, and

Table 2. Normalized percent hybridization (NPH) values

Driver	Tracer				
	D.e.	*E.p.*	*L.s.*	*M.t.*	*C.r.*
D.e.	100	30.6 (1.0)	12.0 (0.5)	17.0 (0.7)	6.0 (0.3)
	100	33.1 (1.1)	13.2 (0.5)	15.3 (0.7)	5.5 (0.3)
E.p.	38.2 (1.1)	100	18.2 (0.7)	23.1 (0.8)	6.5 (0.3)
	35.5 (1.1)	100	17.6 (0.6)	21.1 (0.8)	6.6 (0.3)
L.s.	16.1 (0.7)	16.8 (0.7)	100	43.1 (1.3)	6.2 (0.3)
	15.3 (0.7)	15.4 (0.7)	100	44.1 (1.3)	5.7 (0.3)
M.t.	17.9 (0.7)	21.9 (0.8)	47.5 (1.2)	100	6.9 (0.3)
	15.4 (0.7)	21.9 (0.8)	44.7 (1.1)	100	6.6 (0.3)
C.r.	8.6 (0.5)	7.1 (0.5)	5.9 (0.4)	6.7 (0.5)	100
	7.5 (0.5)	7.0 (0.5)	6.3 (0.4)	6.3 (0.4)	100
L.v.	4.6 (0.4)	3.7 (0.4)	3.4 (0.4)	4.0 (0.4)	5.1 (0.3)
	4.2 (0.4)	4.3 (0.5)	4.0 (0.3)	4.3 (0.4)	4.9 (0.3)
Calf	2.2 (0.3)	1.6 (0.3)	3.1 (0.4)	2.4 (0.4)	3.6 (0.3)
	2.2 (0.4)	2.1 (0.3)	2.6 (0.3)	2.3 (0.3)	3.2 (0.2)

Values for first and second replicates for each tracer are upper and lower values in each cell, respectively. Numbers in brackets are 95% confidence levels based on radioactive counting errors alone. Abbreviations are as in Table 1

also due to the relatively large errors associated with the relatively fewer radioactive counts in the heterologous hybrids.

Rooting the Tree: Choice of Outgroup. The sea biscuit *C. rosaceus* was used to root the evolutionary trees, a choice based on overwhelming morphological evidence (Durham 1955, 1966; Seilacher 1979; Jensen 1981; Smith 1984; Mooi 1987). The fossil record also supports the choice; *Clypeaster* first appears in the fossil record in the mid-Eocene (Ali 1983), whereas the oldest of the four scutellines is only half that age, first appearing in the early Miocene (Durham 1955, 1966; Mooi 1987). *Lytechinus variegatus* was too distant from the clypeasteroids to be included in the phylogenetic analysis.

Computing Topologies. Evolutionary trees were constructed using the least-squares methods provided in PHYLIP version 3.1 (Felsenstein 1988). All data were analyzed with FITCH, which makes no assumptions about the relative rates of evolution in each lineage, and KITSCH, that assumes equal rates of evolution in all branches. In all analyses taxa were added in random order, and global pairwise branch swapping of taxa was performed.

Testing the Molecular Clock

There is no completely rigorous statistical test for rate heterogeneity between lineages (Felsenstein 1984; Powell and Caccone 1990). However, the now relatively commonly used *F*-test (e.g., Bledsoe 1987a; Sheldon 1987; Springer and Kirsch 1989), first used by Rohlf and Sokal (1981) and Felsenstein (1984, 1986) to assess rate variability, is the best available and was employed here. See Felsenstein (1986) for details on applying the test.

Table 3. Uncorrected ΔT_m values

Driver	Tracer				
	D.e.	*E.p.*	*L.s.*	*M.t.*	*C.r.*
D.e.	—	12.3	19.2	19.3	20.9
	—	12.7	17.9	17.4	21.3
Mean	0.0	12.5	18.6	18.4	21.1
E.p.	13.7	—	17.1	15.1	20.7
	14.6	—	17.0	14.9	20.5
Mean	14.2	0.0	17.1	15.0	20.6
L.s.	17.6	14.2	—	11.1	21.1
	17.5	14.6	—	11.8	19.5
Mean	17.6	14.4	0.0	11.5	20.3
M.t.	16.7	13.5	13.3	—	20.4
	17.2	14.6	13.9	—	20.0
Mean	17.0	14.1	13.6	0.0	20.2
C.r.	18.9	16.8	20.7	19.6	—
	18.0	16.6	19.3	17.9	—
Mean	18.5	16.7	20.0	18.8	0.0
L.v.	19.2	17.3	22.2	21.1	21.0
	19.3	18.4	20.8	20.2	20.9
Mean	19.3	17.9	21.5	20.7	21.0

Values for first and second set of hybridizations with each tracer are first and second values in each cell, respectively. Abbreviations are as in Table 1

Results

Extent of Hybridization

Table 2 shows the percent hybridizations, normalized for the extent of reassociation of the homologous hybrid, for each species pair. The raw counts can be found in Marshall (1989).

Melting Curves and Melting Temperatures

One set of melting curves, with error bars indicating the uncertainties in % single-stranded DNA values due to radioactive counting errors, is shown in Fig. 1. Tables 3 (raw values) and 4 (corrected for back mutations) show the differences in melting temperatures (ΔT_m) between each species pair. The com-

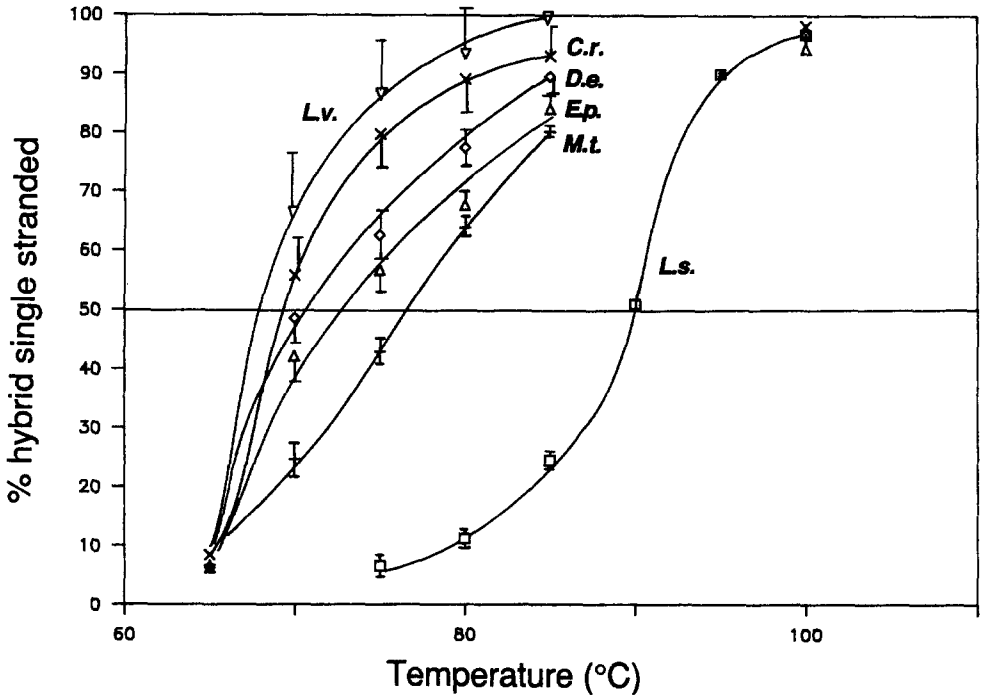

Fig. 1. Melting curves for *Leodia sexiesperforata*. *Leodia sexiesperforata* tracer was hybridized with all other species. Abscissa gives the percent hybrid melted at the given temperature. The error bars are the 95% confidence intervals based on radioactive counting error alone. Abbreviations are as in Table 1.

Table 4. ΔT_m values corrected for multiple hits assuming a ΔT_m of 1°C = 1% mismatch

	Tracer				
Driver	*D.e.*	*E.p.*	*L.s.*	*M.t.*	*C.r.*
D.e.	–	13.4	22.2	22.3	24.5
	–	13.9	20.5	19.8	25.1
Mean	0.0	13.7	21.4	21.1	24.8
E.p.	15.1	–	19.4	16.9	24.2
	16.2	–	19.3	16.6	23.9
Mean	15.7	0.0	19.4	16.8	24.1
L.s.	20.1	15.7	–	12.0	24.8
	19.9	16.2	–	12.8	22.6
Mean	20.0	16.0	0.0	12.4	23.7
M.t.	18.9	14.9	14.6	–	23.8
	19.5	16.2	15.4	–	23.3
Mean	19.2	15.6	15.0	0.0	23.6
C.r.	21.8	19.0	24.2	22.7	–
	20.6	18.8	22.3	20.5	–
Mean	21.2	18.9	23.3	21.6	0.0
L.v.	22.2	19.7	26.3	24.8	24.6
	22.3	21.1	25.4	24.2	24.6
Mean	22.3	20.4	25.9	24.6	24.6

Values for first and second set of hybridizations with each tracer are first and second values in each cell, respectively. Abbreviations are as in Table 1

plete set of melting curves and the raw data used to construct the curves can be found in Marshall (1989). In Fig. 2 the set of melting curves shown in Fig. 1 is replotted so that the abscissa is now a measure of the cumulative counts released at each temperature as a percentage of the total counts available for hybridization (number of counts in homologous hybrid) rather than as a percent of counts in the heterologous hybrid (Fig. 1). The significance of the dramatic difference between these two sets of curves is discussed below.

Median Melting Temperatures

Both for the purposes of phylogenetic reconstruction and the testing for heterogeneity in the rates of DNA sequence divergence it is desirable that the measures of phylogenetic distance are additive. For closely related species, ΔT_m values are additive; however, for species with more divergent DNAs, ΔT_m is not expected to be additive as the T_m's become compressed against the criterion temperature.

The difference in median melting temperatures ($\Delta T_{50}H$, ΔT_mR) is an estimate of the overall divergence between the single-copy DNAs of two species and is usually based on the ΔT_m and NPH values. Specifically, T_mR is the temperature at which 50% of the reactable tracer (defined by the extent of reassociation of the homologous hybrid) has melted. The value can be measured directly from graphs such as Fig. 2. if the NPH is greater than 50% or estimated. When NPH values are less than 50%, the median melting temperature can only be estimated, and Hall et al. (1980) provided the following formula for this purpose:

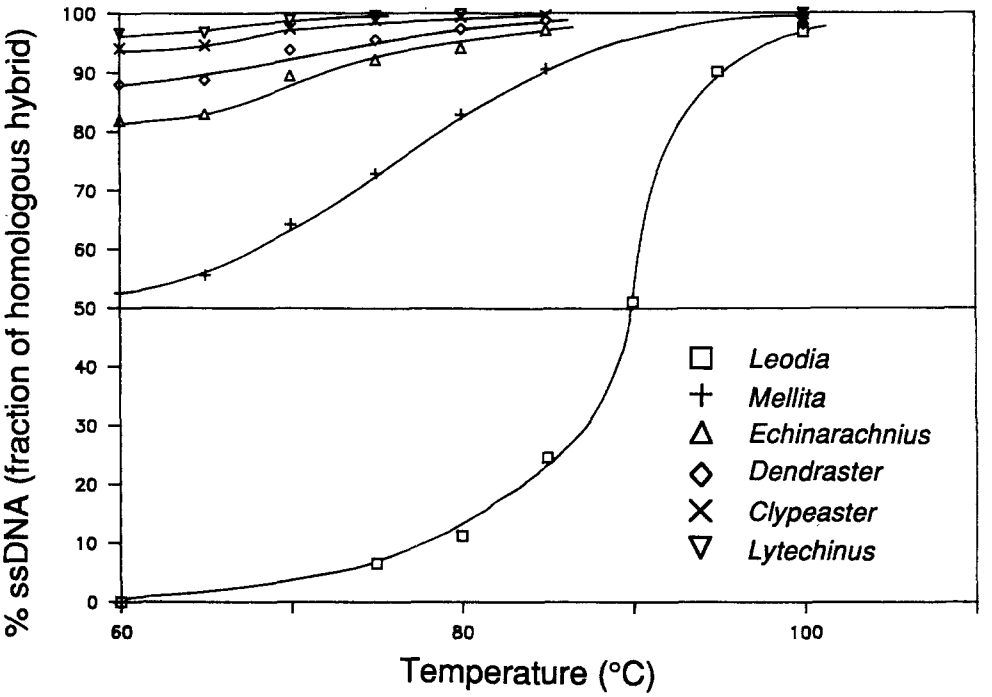

Fig. 2. Melting curves for *Leodia sexiesperforata*. The curves are based on the same data used in Fig. 1, but the abscissa gives the fraction of the hybrid melted, calculated as a percentage of the total tracer available for hybridization (tracer incorporated in homologous hybrid), rather than as a percentage of the counts available in the appropriate heterologous hybrid. Thus, the % single-stranded DNA measured at 60°C gives the NPH for each species pair.

$$\Delta T_mR = [T_m(\text{homologous hybrid}) - T_{\text{criterion}}] \times 50\%/\text{NPH}$$

Note that the T_m's of the heterologous hybrids do not figure in the calculation of ΔT_mR if NPH <50%, and that ΔT_mR becomes solely dependent on the reciprocal of NPH if the homologous T_m's are equal (as is almost the case here). In our study the $T_{\text{criterion}}$ is unknown, and the NPH values are sufficiently low that Hall et al.'s formula does not provide particularly reliable estimates of median melting divergences. However, the compression of the ΔT_m values against the criterion temperature for widely divergent species is severe enough that we have exploited the property that ΔT_mR is essentially solely dependent on 1/NPH and have used 1/NPH as an approximation to ΔT_mR, in addition to ΔT_m, to estimate the phylogeny of the sand dollars. The 1/NPH values can be found in Table 5.

Table 5. Averaged ΔT_m (upper triangle) and 1/NPH values (lower triangle)

	D.e.	*E.p.*	*L.s.*	*M.t.*	*C.r.*
D.e.		14.7 (1.3)	20.7 (1.0)	20.1 (1.5)	23.0 (2.1)
E.p.	0.0293 (0.0028)		17.7 (2.0)	16.2 (0.9)	21.5 (3.0)
L.s.	0.0717 (0.0097)	0.0590 (0.0043)		13.7 (1.6)	23.5 (1.2)
M.t.	0.0613 (0.0047)	0.0455 (0.0017)	0.0224 (0.0009)		22.6 (1.5)
C.r.	0.1495 (0.0300)	0.1473 (0.0064)	0.1662 (0.0077)	0.1511 (0.0058)	

Numbers in brackets are standard deviations. Four values contribute to each mean. ΔT_m values have been corrected for multiple hits assuming a conversion of 1°C = 1% mismatch. Abbreviations are as in Table 1

DNA–DNA Hybridization Phylogeny

Choice of Algorithm

Plots of absolute and percentage errors were made to determine which of the methods, Cavalli-Sforza and Edwards (1967) or Fitch and Margoliash (1967), is the most appropriate for each data set. For each species pair, four measurements of ΔT_m and 1/NPH were made (Table 5 gives the means and standard deviations). The four ingroups are relatively closely related to each other compared with the very distant outgroup, and the absolute and relative errors with and without the outgroup distances were examined to see if the nature of the measurement error changed at very large distances.

The 1/NPH showed a slight positive correlation with absolute SD (correlation coefficient, $r = 0.544$, $r^2 = 0.30$) and no significant correlation with percent SD ($r = 0.108$, $r^2 = 0.01$). If the outgroup was removed, then $r = 0.804$ ($r^2 = 0.71$) with absolute error, and $r = 0.573$ ($r^2 = 0.33$) with relative error. These correlation coefficients suggest that relative

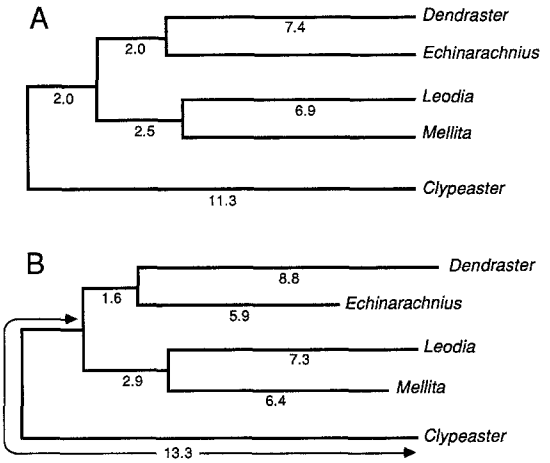

Fig. 3. Phylogeny based on ΔT_m values. **A** Tree generated by assuming a molecular clock (KITSCH) (residual sum of squares = 67.789). **B** Tree generated without the assumption of a molecular clock (FITCH) (residual sum of squares = 39.807). The ΔT_m values are those corrected for back mutation assuming of ΔT_m of 1°C = 1% mismatch between the DNA strands. The Cavalli-Sforza and Edwards (1967) method was used to construct the trees.

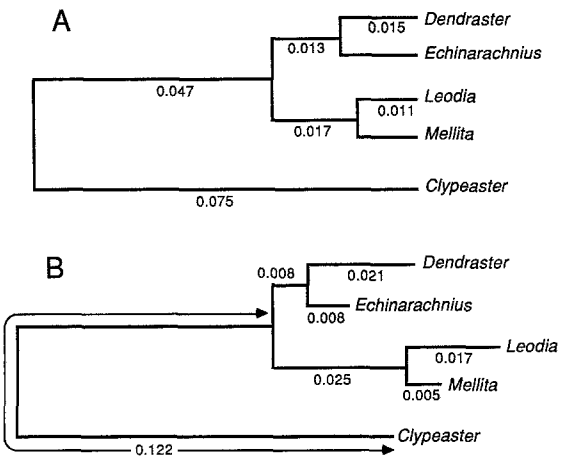

Fig. 4. Phylogeny based on 1/NPH values. **A** Tree generated by assuming a molecular clock (KITSCH) (residual sum of squares = 0.329). **B** Tree generated without the assumption of a molecular clock (FITCH) (residual sum of squares = 0.119). The Fitch and Margoliash (1967) method was used to construct the trees.

Table 6. Tests for rate heterogeneity

Data set	Reciprocals averaged	P[a]	Outgroup removed	Reject[b]
ΔT_m[c]	No	0.0	No	No
	No	0.0	Yes	$0.01 < P < 0.05$
	Yes	0.0	No	$0.01 < P < 0.05$
1/NPH	No	2.0	No	$P < 0.01$
	Yes	2.0	No	$0.01 < P < 0.05$

[a] 0.0 = method of Cavalli-Sforza and Edwards (1967); 2.0 = Fitch and Margoliash (1967)

[b] No = cannot reject null hypothesis of rate homogeneity at $P < 0.05$

[c] Corrected for multiple hits assuming a ΔT_m of 1°C = 1% mismatched base pairs

experimental error is more nearly constant for 1/NPH than absolute error, and that the Fitch and Margoliash (1967) method ($P = 2$) should be used. For ΔT_m (corrected for multiple hits assuming a ΔT_m of 1°C = 1% mismatch) $r = 0.246$ ($r^2 = 0.06$) when correlated with absolute SD, and $r = -0.270$ ($r^2 = 0.07$) when correlated with relative experimental error. The lack of correlation with absolute error suggests Cavalli-Sforza and Edwards' (1967) method should be used, but the lack of any significant negative correlation with relative error is troublesome and probably derives from the fact that ΔT_m (even when corrected for multiple hits) is not expected to behave linearly with phylogenetic distance for large ΔT_m values. If the outgroup is removed, the lack of correlation with absolute error remains ($r = 0.132$, $r^2 = 0.02$), but now a negative correlation with relative error emerges ($r = -0.578$, $r^2 = 0.33$) and Cavalli-Sforza and Edward's (1967) method ($P = 0.0$) is the most appropriate. Almost identical correlation coefficients were obtained when the ΔT_m values were corrected for multiple hits using a ΔT_m of 1°C = 1.7% mismatch.

Phylogenetic Relationships

Some 36 different phylogenetic analyses were performed; the ΔT_m (uncorrected, corrected for 1°C = 1% or 1.7% mismatch) and 1/NPH (the approximation for $\Delta T_m R$) data sets were analyzed with both the Fitch and Margoliash (1967) and Cavalli-Sforza and Edwards (1967) methods, with and without the assumption of a molecular clock, and with and without reciprocal measurements averaged (with or without folded data matrices). All analyses gave the same topology. The ΔT_m trees are shown in Fig. 3, and two of the 1/NPH trees are shown in Fig. 4. Given that ΔT_m distances are expected to be compressed for large phylogenetic distances, the relative branch lengths shown in Fig. 4 (1/NPH, which as an approximation to $\Delta T_m R$ compensates for the compression) are likely to be more realistic than those shown in Fig. 3 (ΔT_m trees).

Rate Heterogeneity

The null hypothesis of constant rates of DNA base substitutions was initially tested for four data sets, ΔT_m and 1/NPH each with and without reciprocal measurements averaged. The results of the F-tests are shown in Table 6 [see Marshall (1989) for details of the calculations]. The null hypothesis of rate constancy was rejected in all analyses except with the unaveraged reciprocal measurements for ΔT_m. The

reciprocal values for the outgroup *Clypeaster* for ΔT_m (Table 4) are in relatively poor agreement, and the differences in these values masks the clear differences observed in the branch lengths within the tree (see Figs. 3 and 4). To test this supposition we tested for rate homogeneity using just the four ingroup taxa, and rate homogeneity could be rejected for ΔT_m.

The 1/NPH values are more reflective of the total genomic differences between species than are ΔT_m's, and thus the 1/NPH phylogeny was used to quantify the degree of rate heterogeneity within the sand dollars. Based on the branch lengths shown in Fig. 4B, *Dendraster* evolved 49% faster, and *Echinarachnius* 49% slower, than the rate of divergence averaged over both genera, and *Leodia* evolved 56% faster and *Mellita* 56% slower than the average rate seen in those species. Note that we have restricted our quantification of the rate heterogeneity to the most closely related species pairs, where the largest NPH values provide the most reliable estimates of $\Delta T_m R$.

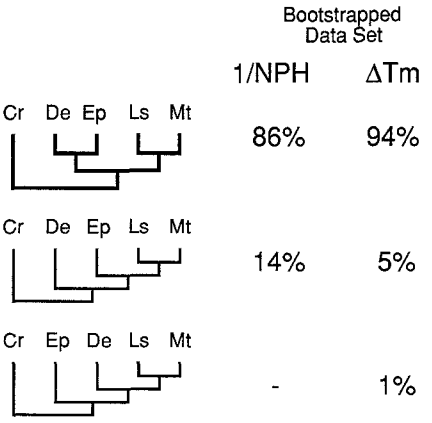

Fig. 5. Frequency of alternative topologies found in the bootstrap analyses. All trees were generated with FITCH (no assumption of a molecular clock). For the 1/NPH data set (Table 5) the algorithm of Fitch and Margoliash (1967) was used; for the ΔT_m data matrix (Table 5) Cavalli-Sforza and Edwards' (1967) method was used. For both data sets 500 pseudoreplicate data sets were analyzed. The phylogeny found in all nonbootstrap analyses is indicated in bold.

Discussion

Robustness of the Phylogeny

The same topology was found in all 36 analyses performed (see Results). There are two possible sources of systematic error that may have influenced the analyses: variations in the length of the hybrids formed and differences in each species AT/GC ratios. However, neither of these appears to be of sufficient magnitude to affect the results of the phylogenetic analysis. Errors introduced by size variation in homologous hybrid lengths (at most 1–2%), or the slight differences in AT/GC ratios (at most 5% error), probably total less than half the random errors associated with measuring radioactivities and experimental manipulations [the reciprocal hybrid 1/NPH values for each species pair differ by up to 10% (data not shown)]. We conclude that the phylogeny is relatively robust.

Jackknifing

As a test of the sensitivity of the phylogenetic analysis to the measurement errors associated with the interspecies distances, each species was removed in turn from the complete data sets to determine whether or not other topologies could be found (Lanyon 1985). Each four-taxon data set was analyzed using FITCH (no assumption of a molecular clock). The Fitch and Margoliash (1967) method was used for the 1/NPH data set (with and without averaging reciprocal distances) and the Cavalli-Sforza and Edwards (1967) method for the ΔT_m data set (with and without averaging reciprocal distances). All analyses gave the same topology as the analyses that used the full complement of taxa.

Bootstrapping

Bootstrapping gives the relative probabilities of alternative topologies within the bounds of the measured experimental error (Krajewski and Dickerman 1990). Figure 5 shows the results of the bootstrap analyses of the 1/NPH and ΔT_m (corrected for multiple hits) data [Marshall (1991) gives detail of the algorithm used]. In all cases the bootstrap analysis most often produced the tree generated by all other analyses. The large uncertainty associated with the *Dendraster/Clypeaster* distance (Table 5) is largely responsible for the high frequency of alternate topologies. The NPH measured between *Dendraster* and *Clypeaster* was measured with relatively high precision (5.5–8.6%, Table 2); however, with such low NPH values even the difference of just 3% in replicate measurements resulted in a large variation in 1/NPH values. The monophyly of *Mellita* and *Leodia* was supported in all 1000 bootstrap trees. Note that undetected sources of random error and all sources of systematic error (e.g., homoplasy) are not taken into account by bootstrapping techniques. Marshall (1991) demonstrates that the nonparametric Mann–Whitney *U*-test is inappropriate for analyzing the robustness of DNA hybridization phylogenies and the test was not applied.

Distance to the Outgroup

The final topology of any tree is strongly dependent on the position of the outgroup. In this study the outgroup, *Clypeaster,* is a considerable distance from the other taxa, and the NPH values between it and the other taxa are low (5.5–8.6%), though the replicate and reciprocal measures are remarkably consistent (Table 2). With such low NPH values,

the possibility that systematic errors due to differences in genome size, single-copy DNA content, or genome organization between *Clypeaster* and the sand dollar becomes more pronounced than for the measurements made between the sand dollars. Thus, the possibility that one of the less-favored topologies shown in Fig. 5 is in fact the correct tree must not be overlooked.

Median Melting Temperatures

Melting curves have been one of the most used tools for phylogenetic analysis in DNA hybridization studies. However, melting curves only give the melting temperature of the fraction of DNA that did hybridize between species but tell us nothing directly of the fraction of DNA that did not (Britten 1990). One could easily form the mistaken impression from the melting curves constructed here (Fig. 1) that not only are all interspecies distances large, but that all species (especially the outgroups) are also rather equidistantly related to the labeled species. This notion is quickly dispelled, however, if one compares the raw melting curves (Fig. 1) with the melting curves adjusted for the extent of hybridization (Fig. 2).

The fact that T_m's become compressed against the criterion temperature for species with divergent DNAs can also be demonstrated by comparing the distances from any sand dollar to *Clypeaster* (first outgroup) and *Lytechinus* (the second outgroup). As estimated by ΔT_m's (Fig. 1), *Lytechinus* appears only slightly more distant than *Clypeaster,* yet paleontological data clearly show that *Clypeaster* diverged from the sand dollars approximately 55 million years (Myr) ago, compared with *Lytechinus,* which diverged some 200 Myr ago (Smith 1984).

Similar Quality ΔT_m and 1/NPH ($\Delta T_m R$) Values

In the recent literature ΔT_m is often favored over $\Delta T_m R$ for the purposes of phylogenetic analyses (e.g., Caccone and Powell 1987, 1989; Sheldon 1987; Springer et al. 1990), though there is also strong support for metrics that incorporate NPH (e.g., $\Delta T_m R$) (Britten 1990; Werman et al. 1990a). In some studies (e.g., Caccone and Powell 1987, 1989) ΔT_m has been measured with an unusually high precision, largely because differences in AT/GC ratios are nullified by melting the hybrid DNAs in 2.4 M TEACL and because the length of hybrid molecules can be measured and thus length corrections made. In all cases where ΔT_m is favored over $\Delta T_m R$, NPH (and therefore $\Delta T_m R$) has been measured with considerably less precision than ΔT_m. In some cases NPH values show very erratic behavior, especially for closely related species. For example, in Caccone and Powell's (1989) hominoid data, the sequence of taxa ordered by decreasing NPH is often very different from the sequence determined by the ΔT_m values, and in some cases heterologous NPH values, even for distantly related species pairs, are greater than 100%.

In contrast to the hominoid data, the NPH values for the clypeasteroids are well behaved. The sequence of taxa is the same for all sets of data regardless of whether they are ordered by decreasing T_m or NPH. For the clypeasteroid data the average percent standard deviation associated with the averaged interspecies distances for the ΔT_m and 1/NPH values are 8.5% and 7.9%, respectively. These values are in close agreement and cannot be distinguished using the Student's *t*-test. ΔT_m was measured with the same degree of reproducibility (as measured by % SD) as in most other studies, despite the large counting errors associated with the melting curves and that no length corrections were made. Secondly, the extent of reassociation values for the homologous hybrids, and by inference the NPH values, have a higher degree of reproducibility for the clypeasteroid data than is typically seen.

Highly Reproducible Extent of Hybridization Values

Under the same experimental conditions all homologous hybrids should incorporate the same proportion of the input counts, regardless of the species involved. Table 7 shows a comparison of the reproducibility of the extent of homologous reassociation values for the few studies where the relevant data are published. Values for the extent of homologous reassociation in this study show a 5–7 times higher reproducibility over the other studies, though Hall et al. (1980) report a similar degree of repeatability for a more limited data set (three measurements).

The greater range of variability in Caccone and Powell's (1987, 1989) data over the clypeasteroid data is the result of at least two differences in the respective experimental procedures [as noted by Caccone and Powell (1989)]. Both concern the consistency in preparation and handling of the DNAs. (1) The DNAs in the hominoid study were derived from several different laboratories, each using different extraction techniques and storage buffers. The storage buffer was a major factor in the highly variable NPH values in this case, and in Caccone and Powell's (1989) primate data anomalously high percent reassociation values are almost always associated with driver DNAs stored in PB. (2) The DNAs in Caccone and Powell's study were often at very different concentrations, and the volume used to carry out the hybridizations were often very differ-

Table 7. Reproducibility of homologous percent hybridization values

Range	Mean ± SD	*n*	Taxa	Data source
70.4–98.5%	77.9% ± 6.7%	24	Cave crickets	Caccone and Powell (1987)
62.0–91.1%	77.4% ± 9.6%	10	Hominoids	Caccone and Powell (1989)
53.3–70.6%	62.0% ± 8.7%	3	Humans	Caccone and Powell (1989)
62.1–65.9%	64.2% ± 1.3%	10	Clypeasteroids	This study

Averaged number of counts incorporated in homologous hybrids, as a percent of input counts, are given with standard deviations (SD); *n* = number of homologous hybridizations performed

ent. A third possible factor is that in Caccone and Powell's protocol the percent reassociations were determined at the first and lighter (95%) of two S1 digestions (the lighter digestion accounts for the higher mean values in the Caccone and Powell data sets). Hall et al. (1980) report a percent reassociation of 64% for an echinoid (*S. purpuratus*) homologous hybrid using the same S1 conditions used in the experiments reported here, i.e., enough S1 nuclease to digest 99% of single-stranded DNA. Perhaps it is difficult to control an incomplete (i.e., 95%) S1 digestion, compared with the 99% digestion. Further experimental data are required to evaluate this possibility.

The high reproducibility of the homologous extent of reassociation values in this study is due to the very careful attention paid to the uniform treatment of the DNAs at all stages of the hybridization experiments; we had the luxury of being able to prepare all DNAs ourselves, and great care was taken to treat each as identically as possible. Equally important, the DNAs were all extracted from the same cell type (sperm), collected, and stored under similar conditions. After the extraction and purification of the DNAs they were all kept at the same concentration, so that all volumes etc. could be kept the same for all hybridizations, S1 digestions, etc.

If great care is taken to keep experimental procedures as constant as possible, it should be possible to produce equal quality ΔT_m and 1/NPH ($\Delta T_m R$) data sets (assuming the group does not have major differences in genome size, etc.) for more widely divergent species than are usually examined using DNA hybridization.

Genome Evolution

Rate Heterogeneity

The four sand dollars show differences of about 50% in the rate of sequence changes in single-copy DNA as compared with the average rate for the group. Rate variation, though only recently investigated, is commonly seen in DNA hybridization data. For example, Sheldon (1987) reported rate variations in herons from 5 to 25% of the average rate seen in the group, Springer and Kirsch (1989) of up to 27% in phalangeriform marsupials, Krajewski (1989) between 30 and 60% in cranes, and Bonner et al. (1980) 85% between Lemuridae and Lorisidae. Our data add to the list of studies that show that single-copy DNA sequences change at quite different rates among relatively closely related species.

Distance to Regular Sea Urchins

The NPH values measured between the five clypeasteroids and the regular sea urchin *L. variegatus* are low, ranging from 1.5 to 2.0% (see Table 2). This corresponds to about 10^7 bp (assuming NPH = 2%). Given the 200 Myr since regular and irregular sea urchins last shared a common ancestor (Smith 1984), any DNA sequences not under strong selective constraints should have diverged sufficiently so as not to hybridize. The sequences that do hybridize thus should represent only highly conserved regions. An estimate of the complexity of the relatively highly conserved gastrula polyribosomal poly(A)$^+$ RNA between different species of the genus *Strongylocentrotus* is 1.7×10^7 bp (Roberts et al. 1985), about twice the complexity seen in the NPH between the regular and clypeasteroid sea urchins. The more conserved fraction of the DNA sequences that code for these polyribosomal poly(A)$^+$ RNAs are probably contributing to the observed NPH between *Lytechinus* and the clypeasteroids. Assuming an average protein is 330 amino acids long, this conserved DNA would code for some 10,000 different proteins, were it all exonic sequences.

Werman et al. (1990b) argue that the conserved regions that hybridize between *Drosophila simulans* and *Drosophila melanogaster* are unlikely to be coding regions because when the nucleotide sequences of coding regions from these two species are compared (e.g., Kwiatowski et al. 1989; Sharp and Li 1989) they show a considerably wider divergence (about 9%) than do the conserved genomic regions that hybridize between the two species in DNA–DNA hybridization experiments (2.7–4.8%). However, the 9% average divergence between the six genes compared [Kwiatowski et al. (1989) and Sharp and Li (1989)] is just for the silent sites, and when the average divergence over all sites in the coding

regions is calculated the average divergence is found to be 3–4%. Thus, the *Drosophila* data are consistent with the notion that exonic regions may be responsible for the small refractory component of genomes that hybridize between widely divergent species.

Plots of NPH Against ΔT_m

Graphs were made of NPH against ΔT_m for each sand dollar tracer against all other sand dollars and *Clypeaster*. As with other DNA hybridization studies, there is a strong negative correlation between NPH and ΔT_m. Correlation coefficients ranged from 0.932 to 0.978 and the slopes of the lines of best fit range from -4.8 to -6.8% NPH/°C ΔT_m. When all data were plotted together the slope was -6.3% NPH/°C ΔT_m, with a correlation coefficient of 0.853.

Similar plots have been constructed for *Drosophila* (-7% NPH/°C ΔT_m; Schulze and Lee 1986), cave crickets (-4 to -5% NPH/°C ΔT_m; Caccone and Powell 1987), and herons [-1 to -2% NPH/°C ΔT_m; Caccone and Powell's (1987) graph of Sheldon's (1987) data]. Clypeasteroids have one of the faster losses of NPH per °C ΔT_m of the limited number of groups studied. Powell and Caccone (1989), Caccone and Powell (1990), and Werman et al. (1990b) discuss the implications of data such as these for our understanding of the dynamics and mechanisms of genome evolution.

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