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Evolution

Rate variation of protein and mitochondrial DNA evolution as revealed by sea urchins separated by the Isthmus of Panama

(speciation/molecular evolution/molecular clock/isozymes/population genetics)

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ABSTRACT **Acceptance of the rough constancy of rates of molecular evolution, averaged over tens of millions of years, is widely used to date the splitting between taxa. However, for the study of speciation a hypothesis of rough constancy over tens of millions of years is of little use. In order to date the splitting of congeneric species within defined ranges of uncertainty, we need to know the variation of evolutionary rates over shorter periods of time. Such estimates of uncertainty are particularly useful if they apply to techniques of molecular comparisons that lend themselves to the assessment of intraspecific variation. We have measured protein divergence by electrophoresis and mitochondrial DNA differentiation by restriction fragment length polymorphism analysis in three pairs of sea urchin species believed to have resulted from the simultaneous fragmentation of ranges of marine species by the emergence of the Isthmus of Panama, about 3 million years ago. Transisthmian isozyme divergence in these pairs varies by an order of magnitude; mitochondrial DNA divergence, on the other hand, is equivalent in all pairs, suggesting that this molecule, assayed by endonucleases, can provide fairly accurate estimates of times since separation in the 3-million-year range.**

One of the reasons for the spectacular increase in the use of molecular approaches to the study of evolution is the widespread belief that molecular divergence between extant taxa can provide information about the time at which they split from each other. This belief is based on the notion that the rate of accumulation of substitutions in biological macromolecules is constant, the so-called "molecular clock" hypothesis (1), which is consistent with the idea that most molecular substitutions are selectively neutral (2). The original postulate that there is a linear relationship between molecular differentiation and time (1, 3) has more recently been succeeded by the concept of an "episodic" or "overdispersed" clock (4, 5), which admits rate variation over short periods of time. Nevertheless, belief in the rough constancy of rates of molecular evolution—at least as averaged over tens of millions of years—prevails; it is widely used to date the splitting between taxa, even when such reconstructions require the reinterpretation of paleontological (6) or geological (7) evidence.

For the study of speciation (as opposed to the emergence of higher categories), however, a hypothesis of rough constancy over tens of millions of years is of little use, because extant species are usually younger than 10 million years. In order to date the splitting of congeneric species within defined ranges of uncertainty, we need to know the variation of evolutionary rates over short periods of time. Such estimates of uncertainty are particularly useful if they apply to methods of molecular comparisons that lend themselves to the assessment of intraspecific variation. Two widely used procedures (reviewed in refs. 8–12) are electrophoretic comparisons of enzymatic proteins and restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA). We have used these techniques to measure protein and mtDNA divergence in three pairs of sea urchin species believed to have been split simultaneously by the emergence of the Isthmus of Panama, about 3 million years ago (13).

The final severance of seawater connections between the Caribbean and the eastern Pacific in the Pliocene is a particularly well-dated recent event in Earth's history. Estimates from change in coiling direction (14) and from extinction (15) of foraminifera, from changes in oxygen and carbon isotope ratios in the two oceans (13), and from biostratigraphic correlations of molluscan fossil faunas (16) are in agreement in dating this event between 2.9 and 3.5 million years ago. Slightly younger dates (2.5–2.8 million years) of South American mammal fossils found in North America (17) and North American ones found in South America (18) confirm that the terrestrial corridor connecting South and North America was completed before the end of the Pliocene. The rise of the isthmus split previously continuous ranges of many marine taxa and has resulted in pairs of closely related marine species, one on each side of Central America. Such species have come to be known as "geminate" (19).

Rate variation in protein evolution has been studied before by the use of geminate pairs (20, 21), with conflicting interpretations of the results (12, 20–24). Here we use data on transisthmian divergence in two classes of macromolecules to provide two conceptually different assessments of the degree of regularity of molecular evolution. The first is based on separate comparisons within each type of molecule; divergence in each geminate pair is contrasted with that of other pairs. The second relies on comparison of mtDNA divergence to protein divergence and asks whether the relative times since separation as determined from each set of data are congruent (25). The first test depends on the assumption that all geminate pairs were split simultaneously; the second test is independent of this assumption and can help distinguish between dissimilarities arising from different dates of separation and dissimilarities that may be due to different rates of evolution.

MATERIALS AND METHODS

Samples of the sea urchins *Eucidaris thouarsi, Echinometra vanbrunti*, and *Diadema mexicanum* were collected from the Pacific coast of Panama and from the Sea of Cortez (Guaymas, Mexico) in the eastern Pacific. *Eucidaris tribuloides, Echinometra viridis, Echinometra lucunter*, and *Diadema antillarum* were sampled from the Atlantic coast of Panama and from Puerto Rico in the Caribbean. Both *Echinometra viridis* and *Echinometra lucunter* are included in the study

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Abbreviation: RFLP, restriction fragment length polymorphism.

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because of uncertainty as to the phylogenetic relationship of the three *Echinometra* species. All collections were made in 1986 and 1987. In their respective oceans these sea urchin species have similar distributions. In addition, they all have planktonic larvae, which increases the probability that the rise of the Panama isthmus may have interrupted gene flow in all three genera simultaneously.

Thirty-four presumptive protein loci were assayed in *Diadema*, 31 in *Echinometra*, and 25 in *Eucidaris*. The following loci were assayed [enzymes included in an earlier study (20) are marked with an asterisk]: in *Diadema*, acid phosphatase (ACPH), glucosidase (GLU), amylases (AM-1,* AM-2*), mannosidase (MAN), β-*N*-acetylgalactosaminidase (βGALA), creatine kinase (CK), esterases (EST-1,* EST-2,* EST-3), fructokinase (FK), fumarase (FUM), glucose-6-phosphate dehydrogenase (G6PDH*), aspartate aminotransferase (GOT), hexokinase (HK*), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP-1, LAP-2*), mannose-6-phosphate isomerase (M6PI*), NAD^+-dependent malic dehydrogenase (MDH-1,* MDH-2*), octanol dehydrogenase (ODH), L-leucyl-L-leucyl-L-leucine peptidase (PEPLLL-1, PEPLLL-2, PEPLLL-3), L-leucyl-L-tyrosine peptidase (PEPLT-1,* PEPLT-2*), phosphoglucose isomerase (PGI*), phosphoglucomutase (PGM-1,* PGM-2*), superoxide dismutase (SOD*), tyrosine aminotransferase (TAT), triosephosphate isomerase (TPI*), and xanthine dehydrogenase (XDH*); in *Echinometra*, ACPH, GLU, AM-1,* AM-2,* βGALA, CK, EST-1, EST-2, EST-3, FK, FUM, G6PDH,* GOT, HK,* IDH, LAP,* M6PI,* MDH-1,* MDH-2,* ODH, PEPLLL-1, PEPLLL-2, PEPLT-1,* PEPLT-2,* PGI,* PGM-1,* PGM-2,* SOD,* TAT, TPI,* and XDH*; in *Eucidaris*, ACPH, GLU, AM-1,* AM-2,* *N*-acetyl-β-glucosaminidase (βGA), βGALA, CK, EST-1,* EST-2,* EST-3,* FK, GOT-1, GOT-2, HK,* LAP,* M6PI,* MDH-1,* MDH-2, ODH, PEPLT,* PGI,* PGM,* SOD,* TPI,* and XDH.* Sample size per population ranged from 17 to 52 individuals. Though an effort was made to assay the same presumptive loci for all genera, technical limitations in obtaining activity and resolution for some enzymes, and possible gene duplications in the evolution of echinoids (26), made this possible for only 23 loci. To determine the degree to which apparent differences in divergence might arise from the inclusion of additional loci with possibly different rates of evolution, measures of divergence were also calculated from the 23 common loci (Table 1).

A subsample of 10–14 individuals per species per location that were analyzed for allozyme variation was also assayed for mtDNA RFLPs by using either purified mtDNA/end-labeling or genomic DNA/filter hybridization methods identical to those published elsewhere (29). All individuals surveyed had their mtDNAs cut with the restriction enzymes *Ava* I, *Bcl* I, *Bgl* I, *Bgl* II, *Cla* I, *Eco*RI, *Hin*dIII, *Pst* I, *Pvu* II, *Sac* I, and *Xho* I. One or two individuals per species per location were assayed with an additional 15 endonucleases (*Asp*700, *Ava* II, *Bam*HI, *Bst*BI, *Bst*EII, *Dra* I, *Eco*RV, *Hin*dII, *Nco* I, *Nde* I, *Sac* II, *Sal* I, *Spe* I, *Stu* I, and *Xba* I) to determine the effect of increasing the proportion of the mtDNA genome sampled on the estimates of sequence divergence between pairs of urchin species. We used restriction endonuclease analysis, rather than direct sequence analysis of one or a small number of genes, to provide estimates of sequence divergence across the entire mtDNA genome and to make our results comparable to the large number of published mtDNA RFLP studies. Intra- and interspecific mtDNA divergence was estimated for each pair of species, using both RFLP data and inferred restriction-site data. Estimates of sequence divergence, whether based on fragment data or site data, and whether derived from 11 or 26 restriction endonucleases, were similar. The two most dissimilar estimates of mtDNA sequence divergence in this study were observed in the comparisons of the Mexico population of *Eucidaris thouarsi* and the Puerto Rico population of *Eucidaris tribuloides*. For fragment data for 11 enzymes the mean sequence divergence (p; ref. 30) equaled 0.0792; for inferred site data for 26 enzymes the mean sequence divergence (D_{xy}; ref. 28) equaled 0.0683. The divergence values reported in Table 2 were calculated from restriction-site data for the 11 endonucleases used to assay all 168 sea urchins surveyed for mtDNA polymorphisms.

RESULTS AND DISCUSSION

Both protein and mtDNA intraspecific genetic distances were very small in these sea urchins (Tables 1 and 2). Transisthmian distances, on the other hand, exceeded intraspecific ones by at least 1 order of magnitude, with one important exception: the protein divergence between Atlantic and Pacific *Diadema* was not significantly larger than differentiation between local populations in each ocean (Fig. 1). As Fig. 2 indicates, the two species of *Diadema* overlap, partially or completely, in allelic frequencies of all 34 sampled loci; in

Table 1. Jackknifed (27) means ± standard errors of Nei's (24) standard genetic distance, based on all available protein loci between congeneric populations of sea urchins

Diadema	*D. antillarum*, Atlantic (Pma)	*D. antillarum*, Atlantic (PR)	*D. mexicanum*, Pacific (Pma)
D. antillarum, Atlantic (PR)	0.013 ± 0.004 (0.015 ± 0.005)		
D. mexicanum, Pacific (Pma)	0.048 ± 0.023 (0.052 ± 0.031)	0.031 ± 0.014 (0.032 ± 0.017)	
D. mexicanum, Pacific (Mex)	0.046 ± 0.026 (0.062 ± 0.038)	0.028 ± 0.014 (0.038 ± 0.022)	013 ± 0.005 (0.014 ± 0.006)
Echinometra	*E. lucunter*, Atlantic (Pma)	*E. lucunter*, Atlantic (PR)	*E. vanbrunti*, Pacific (Pma)
E. lucunter, Atlantic (PR)	0.013 ± 0.004 (0.018 ± 0.006)		
E. vanbrunti, Pacific (Pma)	0.355 ± 0.112 (0.470 ± 0.156)	0.324 ± 0.105 (0.430 ± 0.146)	
E. vanbrunti, Pacific (Mex)	0.354 ± 0.112 (0.471 ± 0.157)	0.331 ± 0.106 (0.442 ± 0.149)	0.016 ± 0.013 (0.022 ± 0.018)
	E. viridis, Atlantic (Pma)	*E. viridis*, Atlantic (PR)	*E. vanbrunti*, Pacific (Pma)
E. viridis, Atlantic (PR)	0.014 ± 0.007 (0.017 ± 0.010)		
E. vanbrunti, Pacific (Pma)	0.523 ± 0.146 (0.574 ± 0.170)	0.532 ± 0.146 (0.583 ± 0.169)	
E. vanbrunti, Pacific (Mex)	0.515 ± 0.145 (0.569 ± 0.169)	0.525 ± 0.144 (0.564 ± 0.165)	0.016 ± 0.013 (0.022 ± 0.018)
Eucidaris	*E. tribuloides*, Atlantic (Pma)	*E. tribuloides*, Atlantic (PR)	*E. thouarsi*, Pacific (Pma)
E. tribuloides, Atlantic (PR)	0.006 ± 0.003 (0.008 ± 0.004)		
E. thouarsi, Pacific (Pma)	0.321 ± 0.118 (0.401 ± 0.139)	0.302 ± 0.112 (0.376 ± 0.133)	
E. thouarsi, Pacific (Mex)	0.323 ± 0.118 (0.404 ± 0.140)	0.302 ± 0.112 (0.376 ± 0.132)	0.002 ± 0.001 (0.002 ± 0.001)

Pma, Panama; Mex, Mexico; PR, Puerto Rico. Values in parentheses were calculated from the 23 loci that were sampled in all three genera.

Table 2. Jackknifed means ± standard errors of average mtDNA nucleotide sequence divergence (D_{xy}; ref. 28) between congeneric populations of sea urchins

Diadema	*D. antillarum*, Atlantic (Pma)	*D. antillarum*, Atlantic (PR)	*D. mexicanum*, Pacific (Pma)
D. antillarum, Atlantic (PR)	0.0056 ± 0.0018		
D. mexicanum, Pacific (Pma)	0.0538 ± 0.0111	0.0526 ± 0.0102	
D. mexicanum, Pacific (Mex)	0.0529 ± 0.0115	0.0518 ± 0.0105	0.0022 ± 0.0010
Echinometra	*E. lucunter*, Atlantic (Pma)	*E. lucunter*, Atlantic (PR)	*E. vanbrunti*, Pacific (Pma)
E. lucunter, Atlantic (PR)	0.0033 ± 0.0011		
E. vanbrunti, Pacific (Pma)	0.0604 ± 0.0121	0.0593 ± 0.0119	
E. vanbrunti, Pacific (Mex)	0.0603 ± 0.0124	0.0593 ± 0.0122	0.0030 ± 0.0010
	E. viridis, Atlantic (Pma)	*E. viridis*, Atlantic (PR)	*E. vanbrunti*, Pacific (Pma)
E. viridis, Atlantic (PR)	0.0049 ± 0.0017		
E. vanbrunti, Pacific (Pma)	0.0824 ± 0.0156	0.0812 ± 0.0154	
E. vanbrunti, Pacific (Mex)	0.0806 ± 0.0150	0.0793 ± 0.0149	0.0030 ± 0.0010
Eucidaris	*E. tribuloides*, Atlantic (Pma)	*E. tribuloides*, Atlantic (PR)	*E. thouarsi*, Pacific (Pma)
E. tribuloides, Atlantic (PR)	0.0022 ± 0.0010		
E. thouarsi, Pacific (Pma)	0.0657 ± 0.0148	0.0653 ± 0.0148	
E. thouarsi, Pacific (Mex)	0.0663 ± 0.0152	0.0659 ± 0.0152	0.0049 ± 0.0025

Pma, Panama; Mex, Mexico; PR, Puerto Rico.

Eucidaris and *Echinometra*, on the other hand, a substantial proportion of loci are fixed (or nearly fixed) for different alleles on the two shores of Central America. Alleles that are represented on only one side of the isthmus either have appeared by mutation after the populations were separated or have been eliminated on the opposite side by natural selection or genetic drift. The differences between the genera in transisthmian Nei's D values are mostly due to these unique alleles that exist in high frequency in only one ocean.

The discrepancy in protein divergence between geminate pairs could be due to one of two causes: either the rates of protein divergence in *Diadema* have been 1 order of magnitude slower than those of the other two urchin genera or its Atlantic and Pacific species were separated more recently. The mtDNA data provide strong evidence in favor of the first alternative. If *D. antillarum* and *D. mexicanum* had been separated 10 times more recently than Atlantic and Pacific species of *Echinometra* or *Eucidaris*, and if each type of molecule evolves at a constant rate, then *Diadema* should have also exhibited reduced transisthmian genetic divergence in mtDNA. This was not the case. mtDNA interspecific divergence in *Diadema* was similar to (and not significantly different from) transisthmian mtDNA divergence in *Echinometra* and *Eucidaris* (Fig. 1). Eight of 11 restriction endonucleases revealed species-specific mtDNA cleavage patterns in *Diadema*, and 9 of 11 in *Eucidaris* and in *Echinometra*. The similarity of transisthmian mtDNA distances in all genera supports our initial assumption of nearly simultaneous splitting of all species pairs by the Isthmus of Panama. The alternative explanation would be that Atlantic and Pacific populations of *Eucidaris* and *Echinometra* were split at a time much earlier than the completion of the isthmus, as belief in a protein clock would suggest. This explanation is less credible because it would require that the rate of mtDNA

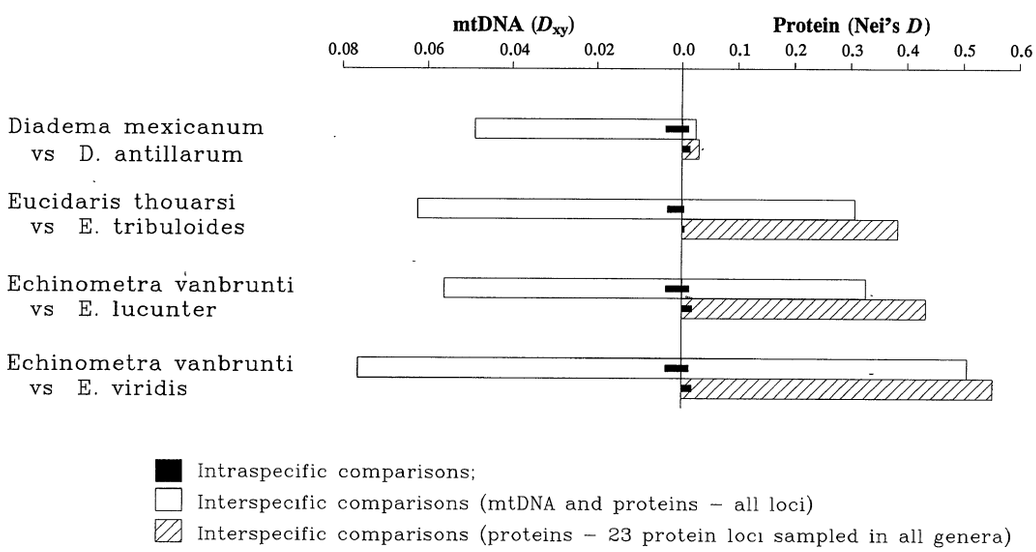

FIG. 1. Mean estimates of divergence between congeneric urchin species, derived from proteins and mtDNA. Transisthmian protein divergence in *Diadema* was not significantly larger than differentiation between populations in each ocean; it was significantly lower than protein divergence in *Eucidaris* and *Echinometra*. All other interspecific (transisthmian) divergence values were significantly larger ($P < 0.05$) than intraspecific values. In addition, interspecific mtDNA genetic distances were not significantly different from one another, nor were interspecific protein distances significantly different between *Eucidaris* and *Echinometra*. Significance remained the same whether calculated on the basis of all available protein loci, or just the 23 loci common to all genera. Means and statistical significance of differences between mean genetic distances were calculated by jackknifing, taking covariance into account (27, 28).

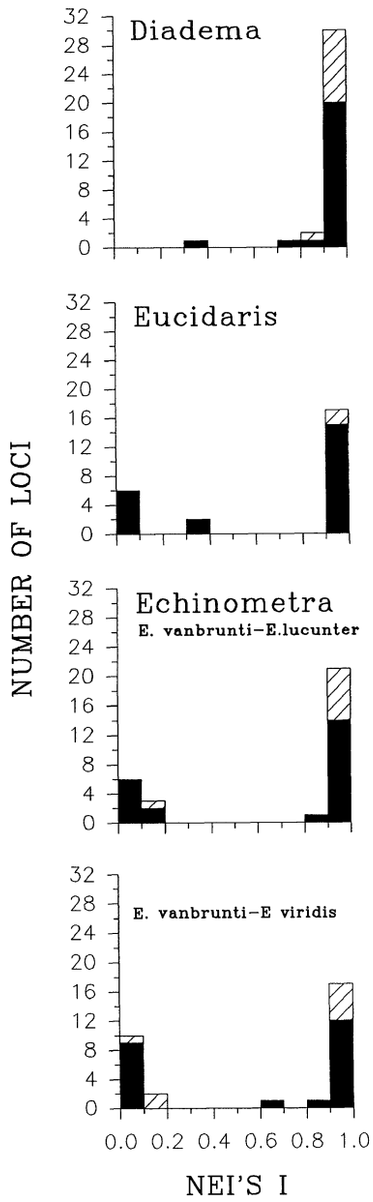

FIG. 2. Number of loci in each interval of Nei's genetic identity I in transisthmian comparisons between congeneric species. Gene frequencies of the populations of each species have been pooled. Solid bars, loci sampled in all genera; hatched bars, loci not sampled in all genera.

evolution in these two genera is unusually slow both as compared to *Diadema* and as compared to other published estimates of mtDNA divergence rates (8, 25).

What might account for the decelerated rate of protein evolution in *Diadema*? Sampling error is an unlikely possibility, given that two independent datum sets, representing *Diadema* populations collected 10 years apart, have combined sample sizes per locus generally exceeding 140 individuals. Error due to sampled loci is also an unlikely explanation for the protein rate variation, given that our estimates of confidence intervals for Nei's D are based on jackknifing over loci. Unequally distributed "hidden variation" is also an unsatisfactory explanation, because intraspecific D values in *Diadema* are no smaller than those observed in *Echinometra* or *Eucidaris*. Heterozygosity in *Diadema* was not lower than in the other two genera either in 1976 (20) or in the present study (data not shown), which also suggests that hidden variation is equal in all genera, and argues against the possibility that genus-specific mutation rates might account for discrepancies in divergence.

D. antillarum suffered mass mortality in 1983, but the reduction in population density did not affect the gene frequencies or the number of effective alleles of this species (31). A population bottleneck would not explain the discrepancy in protein divergence between *Diadema* and the other two genera in any case, because it should have affected mtDNA differentiation even more severely, given that effective population size for mitochondrial genes is one-quarter as large as for nuclear genes (32). Nor can the difference be the result of different generation times (3), because *Diadema* reaches sexual maturity sooner than *Eucidaris* or *Echinometra* (33) and because this parameter should have also affected mtDNA divergence.

We do not know why proteins in the geminate species of *Diadema* have been evolving in parallel for the last 3 million years, while those of the other sea urchins have been diverging. One possibility is that stochastic variation in rates of protein evolution is large enough to produce 10-fold discrepancies over this length of time. According to this explanation, there is nothing special about *Diadema*; any other genus would have an equal probability of showing similar discrepancies in protein divergence. Another possibility is that *Diadema*, because of its ecological generalism relative to the other genera (22), perceives the environments in the two oceans as more similar than do other sea urchins, and its geminate species have thus been under parallel selective regimes. This explanation would require that isozymes are subject to natural selection.

It remains to be seen whether the constant rates of mtDNA evolution found in these three geminate pairs will hold for other taxa separated for the same period of time. However, our comparisons between protein and mtDNA divergence values of species pairs indicate that—regardless of the assumption of simultaneous splitting in all pairs—both kinds of molecules did not evolve at constant rates. If we assume that splitting was in fact simultaneous, as suggested by geological evidence, comparisons of divergence values within each genus with those of other genera suggest that rates of divergence have been variable across the species pairs in proteins but more constant in mtDNA. Note that our mtDNA data do not necessarily suggest that divergence in this molecule is a linear function of time; any monotonic relation between divergence and time would produce equivalent degrees of differentiation between species with coincident times of splitting. An assumption of linearity, coupled with the 3.0-million-year estimate of isthmus completion based on paleooceanographic analysis (13), produces a calibration of the mtDNA molecular clock of 1.8–2.2% sequence divergence per million years, or 1.6–2.1% per million years if the divergence estimates are corrected for intraspecific polymorphism (28).

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