

## Use of Panamanian sea urchins to test the molecular clock

THE 'molecular clock' hypothesis of protein evolution holds that each protein changes at a constant rate, so that the degree of molecular divergence between two species is linearly related to the time for which their lineages have remained separate<sup>1</sup>. This assertion, however, has been challenged repeatedly by authors who discovered taxa and peptides in which the proposed uniformity of molecular evolution did not hold<sup>2,3</sup>, who noted that biochemically and palaeontologically determined dates of separation between lineages conflicted<sup>4,5</sup>, introduced tests that pointed to significant variation in the rates of evolution of the same proteins<sup>6,7</sup>, or dismissed the hypothesis as a confusion of averages with constants<sup>8</sup>. Others have postulated that, although the same proteins evolve at different rates in different lineages, the average amount of molecular change over many proteins is sufficiently uniform to provide approximate dates for the splitting of two lines of descent<sup>9,10</sup>. Here I present evidence from sea urchins separated by the Isthmus of Panama which indicates that even this compromise position is not tenable.

It is important to resolve this issue for two reasons: first, the assumption of a linear relationship between time since separation and degree of molecular divergence has been used extensively to assign dates to the splitting of two lineages on the basis of biochemical evidence<sup>11-15</sup> and second, the proposed constancy of rate of amino acid substitution has frequently been cited as the strongest evidence for the neutral mutation theory, which maintains that the major cause of molecular evolution is random fixation of selectively neutral mutations<sup>16-19</sup>. In spite of one model that tries to account for the phenomenon in terms of natural selection<sup>20</sup>, the most parsimonious theoretical basis for such a uniformity of rates remains that proposed by Kimura<sup>17</sup>. If most amino acid substitutions were neutral, their rate of fixation would equal the mutation rate, and it would be constant over long periods of time, provided that mutations are random events.

Because of the uncertainties involved in assigning fossils to either branch of a phylogenetic tree if they lie close to the node, rate determinations that use dates based on palaeontological evidence have been challenged by both proponents<sup>1,14,15,21</sup> and opponents<sup>22</sup> of the molecular clock. The best way to test the clock hypothesis is to assess the relative amounts of divergence within two or more groups, all of which split into new lines of descent at the same time<sup>1</sup>. Such a situation holds for the species pairs of Panama<sup>11</sup>, where the emergence of the Isthmus in the Pliocene<sup>23,25</sup> fragmented the ranges of many marine species simultaneously. Populations thus separated have become known as geminate species.

There are seven genera of shallow water regular echinoids in the Caribbean, each of which is also represented in the eastern Pacific by populations that have been assigned a different specific name. On morphological grounds the Atlantic and eastern Pacific members of each genus are presumed to comprise geminate pairs<sup>26</sup>. I compared three pairs: *Diadema antillarum* (Caribbean (C)) and *Diadema mexicanum* (Pacific (P)); *Eucidaris tribuloides* (C) and *Eucidaris thouarsi* (P); *Echinometra lucunter* (C) and *Echinometra vanbrunti* (P). *Echinometra viridis*, a species sympatric with *E. lucunter*, was also included, because of the uncertainty as to which of the two is the true geminate of their Pacific counterpart. Enzymatic proteins were compared by electrophoresis; 18 presumptive loci (15 for *Eucidaris*) were sampled for two populations per species. Trans-isthmian divergence is measured relative to differentiation between conspecific population on the same coast. Such a calibration corrects for the possibility that electrophoretically undetected heterogeneity<sup>27</sup> may be unequally distributed among the genera or that the enzymes assayed are a nonrandom sample of the existing rates of molecular evolution. Differences are quantified by Nei's<sup>28</sup> standard genetic distance.

The clock hypothesis predicts that the extent of molecular differentiation in all pairs should be equivalent. Clearly, this is not the case (Table 1). Pacific populations of *Diadema* have diverged from their Atlantic counterparts no more than they have from populations on the same coast. *Eucidaris* and *Echinometra*, on the other hand, exhibit trans-isthmian distances 16 and 37 times greater than intraspecific ones. Even if we take the conservative stand that intraspecific distances are roughly the same for all genera, we find that the values of Nei's index for inter-oceanic comparisons of *Echinometra* are more than 20 times larger than those for *Diadema*. This conclusion holds whether *E. vanbrunti* is compared with either *E. lucunter* or *E. viridis*.

It is unlikely that the determined dissimilarities in divergence between geminates in the three genera are artefacts of the limited resolving power of electrophoresis. If 'hidden variation' in *Diadema* were 20 times lower than in *Echinometra*, we would expect the former also to exhibit lower values of intraspecific differentiation and heterozygosity. No such trend is apparent (Tables 1, 2).

The 20-fold difference between *Echinometra* and *Diadema* is also too great to be the result of different times of isolation during the gradual closing of the portals connecting the two oceans. All three genera produce planktonic larvae<sup>29</sup>, and cessation of gene flow between their members must have been almost simultaneous. Furthermore, although the duration of the actual geological event is unknown, there is a range of estimates for the date of final completion of the land bridge between North and South America; the highest currently accepted estimate is 3.5-5.7 Myr ago<sup>23,30</sup>, and the lowest about 2 Myr ago<sup>24,25</sup>. If we place the separation of *E. vanbrunti* from *E. lucunter* (or *E. viridis*) at this date and accept that divergence is linearly related to time, the analogous event in *Diadema* would have to be dated at 100,000 to 285,000 yr ago, when the land bridge between North and South America was continuous<sup>25</sup>. Similarly, the postulate that *Diadema* split at the time the Isthmus rose would require the speciation event in *Echinometra* to have occurred between the Eocene and the Cretaceous. Such an early origin of the extant species, whether due to the emergence of the Isthmus or some other geological occurrence, is unlikely as, despite a good echinoid fossil record, the genus is unknown before the Pliocene in the eastern Pacific<sup>31</sup> and the Oligocene in the Caribbean<sup>32</sup>.

The disparity of the genetic distance values is also too great to be due to the stochastic variation allowed by proponents of the clock<sup>1,19</sup>. Nor can it be the result of generation time<sup>33</sup>, as *Diadema* reaches sexual maturity sooner than *Eucidaris* or *Echinometra*<sup>34</sup>.

The possibility that the extant species of *Echinometra* and *Eucidaris* may have speciated after the rise of the Isthmus from true geminates which subsequently became extinct would not redeem the clock hypothesis as molecular clocks are based on the assumption that divergence is dependent on the time two lineages have remained separate and not on the number of speciation events in each.

Different amounts of divergence between the species of *Diadema*, *Eucidaris* and *Echinometra* must, therefore, reflect dissimilar rates of protein differentiation, and argue against the molecular clock hypothesis. West (in preparation) has reached the same conclusion on the basis of allozymic differentiation in four geminate species pairs of Panamanian crabs.

The results could still be compatible with the neutral mutation theory if they reflected differences between the genera in mutation rates or in histories of population size. Both these postulates, however, would conflict with the predictions of the same theory regarding the heterozygosity values of the populations involved.

Many models<sup>35-37</sup> predict that variability of neutral alleles is a function of the mutation rate. If *Diadema* has a mutation rate 20 times lower than *Echinometra*, it should also display less gene diversity. This is not the case (Table 2). Average heterozygosity

**Table 1** Nei's standard genetic distance (D) between populations in each genus  $\pm$  s.e.

Species	Locality	Atlantic				Pacific
		<i>E. lucunter</i> Maria Chiquita	B. del Toro	San Blas Is	<i>E. viridis</i> B. del Toro	<i>E. vanbrunti</i> P. Paitilla
<i>E. lucunter</i>	B. del Toro	0.009 $\pm$ 0.004 (0.012 $\pm$ 0.006)				
<i>E. viridis</i>	San Blas Is	0.117 $\pm$ 0.075 (0.180 $\pm$ 0.121)	0.109 $\pm$ 0.073 (0.169 $\pm$ 0.119)			
	B. del Toro	0.117 $\pm$ 0.073 (0.177 $\pm$ 0.120)	0.111 $\pm$ 0.072 (0.172 $\pm$ 0.119)	0.007 $\pm$ 0.003 (0.008 $\pm$ 0.005)		
<i>E. vanbrunti</i>	P. Paitilla	0.557 $\pm$ 0.209 (0.655 $\pm$ 0.288)	0.531 $\pm$ 0.203 (0.649 $\pm$ 0.290)	0.620 $\pm$ 0.218 (0.771 $\pm$ 0.303)	0.612 $\pm$ 0.214 (0.790 $\pm$ 0.304)	
	Isla Uva	0.561 $\pm$ 0.213 (0.658 $\pm$ 0.295)	0.547 $\pm$ 0.209 (0.672 $\pm$ 0.299)	0.666 $\pm$ 0.233 (0.847 $\pm$ 0.333)	0.653 $\pm$ 0.225 (0.854 $\pm$ 0.328)	0.021 $\pm$ 0.011 (0.032 $\pm$ 0.018)
<i>E. lucunter</i> - <i>E. vanbrunti</i>						
Mean intraspecific genetic distance:		0.015 (0.022)				
Mean transisthmian genetic distance:		0.549 (0.659)				
<i>E. viridis</i> - <i>E. vanbrunti</i>						
Mean intraspecific genetic distance:		0.014 (0.010)				
Mean transisthmian genetic distance:		0.638 (0.816)				
<i>E. tribuloides</i>						
				San Blas Is	B. del Toro	<i>E. thouarsi</i> P. Paitilla
<i>E. tribuloides</i>	B. del Toro			0.016 $\pm$ 0.014 (0.021 $\pm$ 0.018)		
<i>E. thouarsi</i>	P. Paitilla			0.292 $\pm$ 0.143 (0.400 $\pm$ 0.193)	0.307 $\pm$ 0.150 (0.419 $\pm$ 0.202)	
	Isla Uva			0.357 $\pm$ 0.166 (0.480 $\pm$ 0.221)	0.360 $\pm$ 0.166 (0.482 $\pm$ 0.221)	0.024 $\pm$ 0.012 (0.028 $\pm$ 0.015)
Mean intraspecific genetic distance:		0.020 (0.025)				
Mean transisthmian genetic distance:		0.329 (0.445)				
<i>D. antillarum</i>						
				Ft Randolph	B. del Toro	<i>D. mexicanum</i> Isla Uraba
<i>D. antillarum</i>	B. del Toro			0.036 $\pm$ 0.016 (0.038 $\pm$ 0.019)		
<i>D. mexicanum</i>	Is Uraba			0.040 $\pm$ 0.026 (0.052 $\pm$ 0.040)	0.016 $\pm$ 0.007 (0.023 $\pm$ 0.011)	
	Isla Uva			0.039 $\pm$ 0.017 (0.046 $\pm$ 0.023)	0.008 $\pm$ 0.004 (0.012 $\pm$ 0.007)	0.015 $\pm$ 0.007 (0.024 $\pm$ 0.010)
Mean intraspecific genetic distance:		0.026 (0.031)				
Mean transisthmian genetic distance:		0.026 (0.033)				

Values in parentheses are those obtained from an analysis restricted to the 12 loci common to all three genera. Electrophoresis was carried out on 11% starch for all enzymes except amylase; 7% acrylamide gels were used for the latter. Standard histochemical techniques were used for staining. Collections were made at: Fort Randolph, Canal Zone (*D. antillarum*); Bocas del Toro, Republic of Panama (*D. antillarum*, *E. tribuloides*, *E. viridis*, *E. lucunter*); Maria Chiquita, Republic of Panama (*E. lucunter*); San Blas Islands, Republic of Panama (*E. viridis*, *E. tribuloides*); Isla Uraba, Bay of Panama (*D. mexicanum*); Punta Paitilla, Bay of Panama (*E. thouarsi*, *E. vanbrunti*); Isla Uva, Gulf of Chiriqui (*D. mexicanum*, *E. thouarsi*, *E. vanbrunti*). Loci examined for each genus were: *Diadema*: phosphoglucosylase (PGM); PGM-1, PGM-2; phosphoglucose isomerase (PGI); hexokinase (HK); peptidases (Pep): Pep-1, Pep-2, mannose-6-phosphate isomerase (M6PI); NAD-dependent malate dehydrogenase (MDH); MDH-1, MDH-2, triose phosphate isomerase (TPI); leucine amino peptidase (LAP); amylase (Am): Am-1, Am-2; tetrazolium oxidase (TO); glucose-6-phosphate dehydrogenase (G6PDH); xanthine dehydrogenase (XDH); esterases (Est): Est-1, Est-2. *Echinometra*: PGM-1, PGM-2, PGI, HK, Pep-1, Pep-2, M6PI, MDH-1, MDH-2, TPI, Am-1, Am-2, Am-3, TO, G6PDH, XDH, LAP-1, LAP-2. *Eucidaris*: PGM-1, PGI, HK, Pep-1, M6PI, MDH-1, TPI, LAP, AM-1, AM-2, TO, XDH, Est-1, Est-2, Est-3. Sample sizes per locus (number of alleles) ranged from 36 to 134 (most above 90), with one, PGM-1, *E. thouarsi*, Isla Uva, represented by only six. See ref 39 for details of the methods.

**Table 2** Average heterozygosity (H) (Nei's<sup>28</sup> gene diversity) of populations studied  $\pm$  s.e.

Species	Atlantic		Species	Pacific	
	Locality	H		Locality	H
<i>E. lucunter</i>	M. Chiquita	0.254 $\pm$ 0.058 (0.278 $\pm$ 0.080)	<i>E. vanbrunti</i>	Paitilla	0.216 $\pm$ 0.056 (0.278 $\pm$ 0.076)
	B. del Toro	0.259 $\pm$ 0.058 (0.291 $\pm$ 0.081)		Isla Uva	0.182 $\pm$ 0.052 (0.229 $\pm$ 0.073)
<i>E. viridis</i>	San Blas	0.202 $\pm$ 0.065 (0.231 $\pm$ 0.093)			
	B. del Toro	0.183 $\pm$ 0.061 (0.232 $\pm$ 0.087)			
<i>E. tribuloides</i>	San Blas	0.192 $\pm$ 0.045 (0.214 $\pm$ 0.050)	<i>E. thouarsi</i>	Paitilla	0.243 $\pm$ 0.051 (0.288 $\pm$ 0.056)
	B. del Toro	0.202 $\pm$ 0.048 (0.221 $\pm$ 0.053)		Isla Uva	0.111 $\pm$ 0.036 (0.115 $\pm$ 0.040)
<i>D. antillarum</i>	Ft Randolph	0.216 $\pm$ 0.056 (0.276 $\pm$ 0.072)	<i>D. mexicanum</i>	Uraba	0.201 $\pm$ 0.056 (0.249 $\pm$ 0.072)
	B. del Toro	0.162 $\pm$ 0.051 (0.181 $\pm$ 0.068)		Isla Uva	0.184 $\pm$ 0.049 (0.217 $\pm$ 0.063)

Values in parentheses are those obtained from an analysis restricted to the 12 loci common to all three genera.

values are very similar in populations belonging to the two genera. A severe population restriction more recent than  $10^7$  generations ago in *Echinometra* but not *Diadema* could also result in dissimilar rates of divergence compatible with the neutral mutation theory<sup>38</sup>. However, if the higher rates of divergence in *Echinometra* were the result of such a bottleneck, neutral theory would expect its populations in at least one ocean to exhibit lower levels of heterozygosity than those of *Diadema*<sup>38</sup>. This is not so (Table 2).

I conclude that the molecular clock hypothesis does not hold for the Panamian echinoids, and that their unequal rates of protein divergence with similar levels of heterozygosity are in direct opposition to the predictions of at least one model based on the neutral mutation theory. The most plausible explanation for the observed discrepancies is that molecules have been evolving under the influence of natural selection.

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