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CORRELATED EVOLUTIONARY DIVERGENCE OF EGG SIZE AND A MITOCHONDRIAL PROTEIN ACROSS THE ISTHMUS OF PANAMA

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Abstract.—An explicit assumption of studies that employ a mitochondrial DNA (mtDNA) molecular clock is that mtDNA evolves independently of morphology. Here we report a very strong correlation between egg size divergence and cytochrome *c* oxidase-1 (CO1) amino acid sequence divergence among sister species of bivalve molluscs separated by the Central American Isthmus (i.e., ''geminate'' species). Analyses of the molecular data reveal that CO1 sequences likely did not diverge as a function of time or evolve in response to positive natural selection. Given that an excess of CO1 amino acid polymorphism exists within species (as expected if most mutations are only slightly deleterious), a third hypothesis is that reductions in effective population size could simultaneously increase the fixation rate of nearly neutral mtDNA polymorphisms and in some way also facilitate egg size evolution. The remarkable strength of the relationship between egg size and CO1 amino acid sequence demonstrates that, even in the absence of an obvious functional relationship or clock-like evolution, the amounts of molecular and morphological change can be tightly correlated, and therefore may reflect common processes. Accordingly, the assumption that the evolutionary divergence of molecules and morphology are independent must always be carefully examined.

Key words.—Effective population size, geminate species, life-history evolution, molecular clock, nearly neutral evolution.

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The molecular clock is frequently used to investigate the tempo of evolution among organisms, particularly those that lack an informative fossil record (e.g., Bermingham et al. 1992; Patton et al. 1994; Doolittle et al. 1996; Wray et al. 1996; Hart et al. 1997; Voelker 1999). Because genes used in molecular clock studies are generally not directly involved in morphogenesis, rates of molecular and morphological change are usually assumed to be evolutionarily decoupled. Several reviews support this generalization (e.g., Zuckerkandl and Pauling 1965; Wilson et al. 1977; Kimura 1983; Wilson 1991; Sheldon and Bledsoe 1993; Avise 1994), although these studies have been criticized on the grounds that they often focus on taxa that exhibit unusual patterns of divergence in either morphological or molecular characters (Omland 1997). A handful of studies in which there is no a priori expectation of decoupled molecular and morphological evolutionary change, however, suggest that molecular and morphological evolution may at times be related (Bousquet et al. 1992; Smith et al. 1992; Savard et al. 1993; Omland 1994, 1997; Bronham 2002).

Ideally, to test for correlations in evolutionary rates, comparisons should involve characterization of both molecular and morphological divergences in taxa that provide multiple phylogenetically independent comparisons (Bronham 2002). Geminate sister species pairs separated by the Isthmus of Panama are one such model system, providing multiple, independent, natural experiments for examining rates and patterns of evolution in contrasting environments (Lessios 1979; Vawter et al. 1980; Bermingham and Lessios 1993; Knowlton et al. 1993; Cunningham and Collins 1994; T. Collins 1996; Bermingham et al. 1997; Knowlton and Weigt 1998). Because the timing of final seaway closure is so well characterized in the geological record (Keigwin 1982; Duque-Caro 1990; Coates and Obando 1996), a minimum time of divergence of 3.1–3.5 million years ago may be inferred for geminate species pairs. Recent comparative molecular studies, however, indicate a complex historical pattern of geographic isolation among geminate species pairs: some geminate pairs are more divergent than others (e.g., Bermingham and Lessios 1993; Knowlton and Weigt 1998), suggesting that some species pairs were formed prior to final seaway closure. Studies of morphological evolution across the Isthmus likewise have not always found consistent patterns of transisthmian divergence (e.g., Lessios 1981; Lessios and Weinberg 1994; Marko and Jackson 2001).

In this paper, we focus on the evolutionary relationship between egg size and mitochondrial cytochrome *c* oxidase 1 (CO1) DNA sequences between marine bivalve molluscs (Family: Arcidae) separated by the Isthmus of Panama. After the rise of the Isthmus approximately 3.1–3.5 million years ago (reviewed by Coates and Obando 1996), environmental changes resulted in dramatic alterations to ocean productivity that coincided with benthic faunal turnover in the western Atlantic (WA) and divergence of life-history characters on either side of the Isthmus (Stanley 1986; Vermeij and Petuch 1986; Lessios 1990; Allmon et al. 1993; Jackson et al. 1993; Jackson and Herrera 1999). Today, the tropical eastern Pacific (EP) has substantially higher productivity than the tropical WA (Bishop and Marra 1984; Allmon et al. 1996) whereas, before closure of the seaway between the WA and the EP, oceanographic conditions in the entire tropical American region are believed to have been similar to those found in the

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EP today (Glynn 1972; L. Collins 1996). Among modern echinoderm geminates, Pacific species have smaller eggs than their Caribbean geminates (Lessios 1990); this difference has been attributed to natural selection acting on trade-offs between egg size and fecundity in response to contrasting planktonic larval feeding conditions following the rise of the Isthmus (Lessios 1990; Jackson and Herrera 1999). In agreement with this trend among echinoderms, eggs are also larger in the WA member of five of five geminate species pairs of bivalves in the family Arcidae (Moran, unpubl. ms.).

We investigated the relationship between molecular and morphological evolution in geminate arcid bivalves by comparing transisthmian egg size differences to CO1 sequence divergences between five geminate species pairs. Our results demonstrate that egg size and amino acid differences are highly correlated. We then used the molecular data to address two hypotheses as to why these traits exhibit correlated divergences. Last, we considered how correlations between functionally unrelated molecular and morphological traits can provide insights for understanding the tempo and mode of life-history changes on either side of the Isthmus.

Materials and Methods

Egg sizes of geminate species were determined by collecting adults of each species in the field (authors may be contacted for sample localities), stripping ripe oocytes from female gonads, and estimating oocyte volume from the two maximum diameters ($v = 4/3\pi a^2 c$). The sizes of 15 ripe oocytes from three to six females of each species were measured. Egg size divergence for each geminate pair was calculated by subtracting the mean egg size of EP species from the mean egg size of their WA geminates.

Genomic DNA was extracted from each specimen by proteinase K digestion of tissue in 2X CTAB (2% hexadecyltrimethylamonium-bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris, 0.2% β-mercaptoethanol, pH 8.0) for three hours to overnight followed by two chloroform:isoamyl alcohol (24:1) extractions and isopropanol precipitation.

Using 1–2 μL of the extraction as template, we amplified partial CO1 sequences with universal primers (Folmer et al. 1994) and standard amplification conditions (Marko 1998). Amplification was achieved with 35 cycles of 94°C for 30 sec, 42°C for 30 sec, a 30-sec ramp to 72°, and 1 min and 30 sec at 72°C. Sequencing of amplification products in both directions was accomplished with an automated sequencing system (Applied Biosystems, Foster City, CA).

Sequences were easily aligned due to the absence of insertions and deletions, and were then analyzed phylogenetically using maximum likelihood (ML) and maximum parsimony (MP). All searches were conducted in PAUP* version 4.0 (Swofford 2001). In the MP analysis, all nucleotides were weighted equally. The reliability of the MP tree was also characterized by resampling the dataset 500 times with the nonparametric bootstrap. Because substitution model choice in ML analyses can significantly affect branch-length estimates and other analyses (Zhang 1999; Buckley et al. 2001; Posada and Crandall 2001), we used a hierarchical likelihood ratio test (HLRT; Huelsenbeck and Crandall 1998) implemented with Modeltest version 3.04 (Posada and Crandall 1998; Posada and Crandall 2001) to choose the best fitting substitution model in ML analyses. We also compared results under models chosen with HLRTs to those generated with the arbitrarily chosen Hasegawa-Kishino-Yano model (Hasegawa et al. 1985) with rate heterogeneity among nucleotide sites (Yang 1994, 1996). For each ML analysis, model parameters were inferred with likelihood from neighbor-joining tree topologies (Saitou and Nei 1987) generated from Jukes-Cantor genetic distances (Jukes and Cantor 1969). All phylogenetic trees were rooted with an additional sequence obtained from the confamilial *Anadara grandis*. For each geminate pair we also calculated nucleotide sequence divergences separately at replacement and synonymous nucleotide sites. ML model parameters were separately inferred for replacement and synonymous sites from neighbor-joining tree topologies. To investigate variability in substitution rates among sequences, we used the likelihood ratio test (LRT; Felsenstein 1981). The test statistic was calculated by taking two times the difference between the log-likelihood values obtained under the constraint of a molecular clock and the log-likelihood value under no constraint of equal substitution rates. The statistic was then evaluated with a chi-square test with $n - 2$ degrees of freedom (n = number of terminal taxa or sequences). Although the use of a chi-square distribution for the LRT has been questioned (Goldman 1993), simulations show that the chi-square distribution is appropriate in most cases (Yang et al. 1995). All sequences are deposited in GenBank (accession nos. AF253475–AF253494 and AF345641–AF345647).

Results and Discussion

Phylogenetic Relationships and Patterns of Transisthmian Divergence

For all 603 nucleotide sites, a submodel of the General Time Reversible model (Rodriguez et al. 1990) in which all transversions occur at an equal rate, each type of transition has a unique rate, and rate heterogeneity exists among nucleotide sites (TrN + G) was chosen with a HLRT. Using this substitution model, ML produced a tree in which all five arcid geminate pairs formed sister-taxa (Fig. 1). An ML tree constructed under the assumptions of the HKY + G model and the best tree found with unweighted MP were both identical to the tree in Figure 1. Sister-group relationships for all geminate pairs were strongly supported by MP bootstrap values, exceeding 98% for all five geminate pairs (Fig. 1).

Sequence divergences (calculated with models chosen with HLRTs) across all nucleotide sites varied substantially among geminate pairs (Fig. 1), as has been reported in other surveys of geminates, suggesting that either arcid geminates did not all diverge at the same time (e.g., Knowlton et al. 1993; Knowlton and Weigt 1998; Bermingham et al. 1997) or substantial rate heterogeneity exists among transisthmian lineages. In other taxa where analyses of multiple geminate pairs exhibit a wide range of sequence divergences, the least divergent pair is assumed to provide the best estimate of rates of sequence divergence (e.g., Knowlton and Weigt 1998). Using the CO1 divergence from the *Arcopsis* pair, which exhibits the smallest divergence across all sites (15.9%), an isolation time of 3.1 million years ago results in a maximum

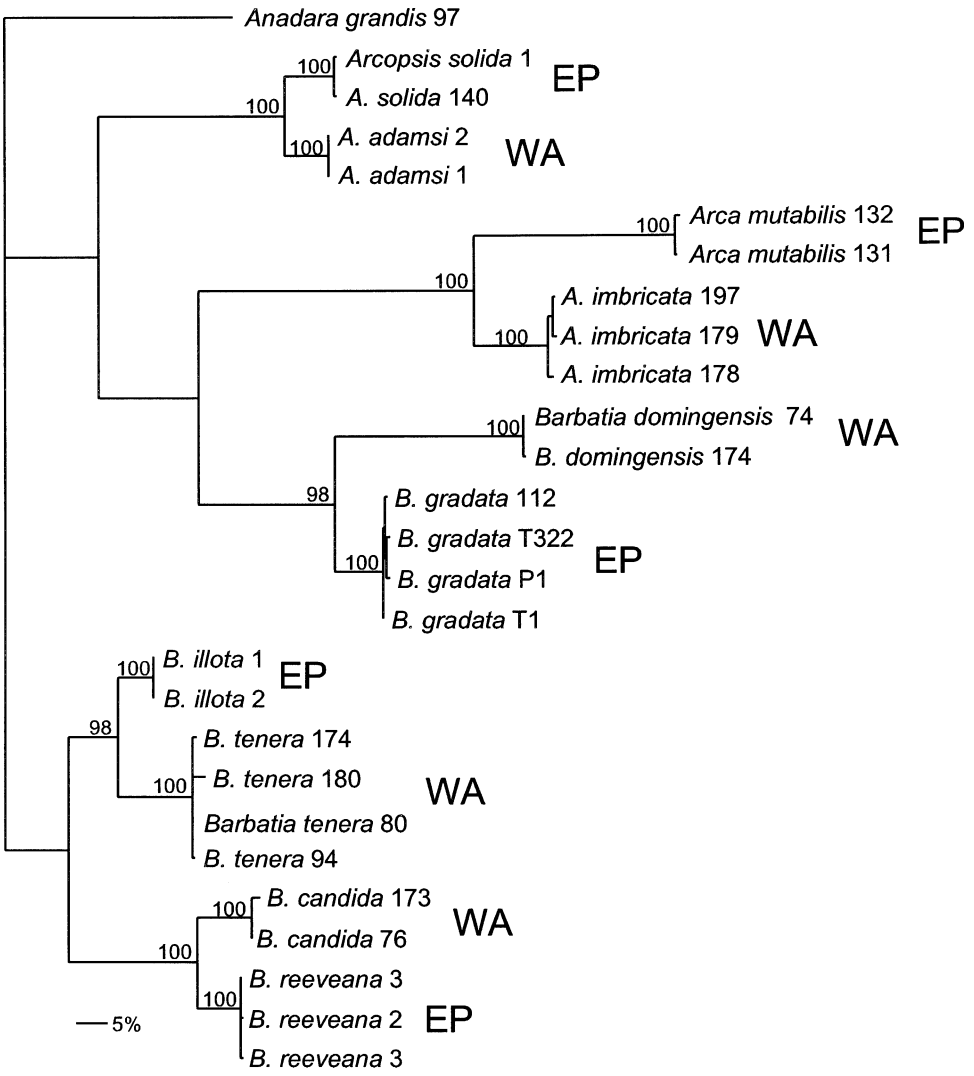

FIG. 1. Phylogenetic relationships of cytochrome *c* oxidase I sequences from arcid bivalve geminate species pairs (tree topology from maximum-likelihood analysis of all sites under the TrN + G model). Numbers over internal branches are bootstrap percentages from 500 maximum-parsimony searches (see Materials and Methods). Only bootstrap values >50% are shown. EP and WA refer to eastern Pacific and western Atlantic, respectively. Scale bar represents 5% divergence.

rate of CO1 divergence of 5.1% per one million years. Application of this rate to the other four geminate pairs results in a broad range of divergence times spanning the last 10 million years (3.4 million years for *Barbatia candida*/*B. reeveana*, 5.1 million years for *B. illota*/*B. tenera*, 8.1 million years for *B. gradata*/*B. domingensis*, and 9.4 million years for *Arca mutabilis*/*A. imbricata*). This isthmian-based calibration, however, likely overestimates the actual divergence rate given that both *A. mutabilis* and *A. imbricata* can be distinguished in the fossil record as far back as 16 million years (Marko and Jackson 2001). Unlike snapping shrimp geminates (Knowlton et al. 1993; Knowlton and Weigt 1998), arcid geminate divergences appear unrelated to habitat or bathymetry. For example, the least divergent pair (*Arcopsis solida*/*A. adamis*) is often found in syntopy with the second most divergent pair (*Barbatia gradata*/*B. domingensis*) in shallow water and intertidal habitats.

Correlation Analyses

Across all sites, we found a positive but marginally nonsignificant correlation between sequence divergence and differences in egg size ($r = 0.874$, df = 3, $P > 0.05$). However, separate consideration of replacement and synonymous site divergences revealed that the positive correlation observed across all sites was driven largely by divergences at replacement sites. Under the TrN + G substitution model (chosen for replacement sites with a HLRT), CO1 replacement site and egg size divergences were significantly correlated ($r = 0.927$, df = 3, $P < 0.05$). Comparing the mean number of amino acid differences between geminates revealed an even stronger relationship: the average number of amino acid differences explains 99% of the variation in egg size divergence (Fig. 2a). In contrast, there was a weak (and nonsignificant) relationship between egg size divergence and CO1 divergence at synonymous nucleotide sites (HKY model, selected

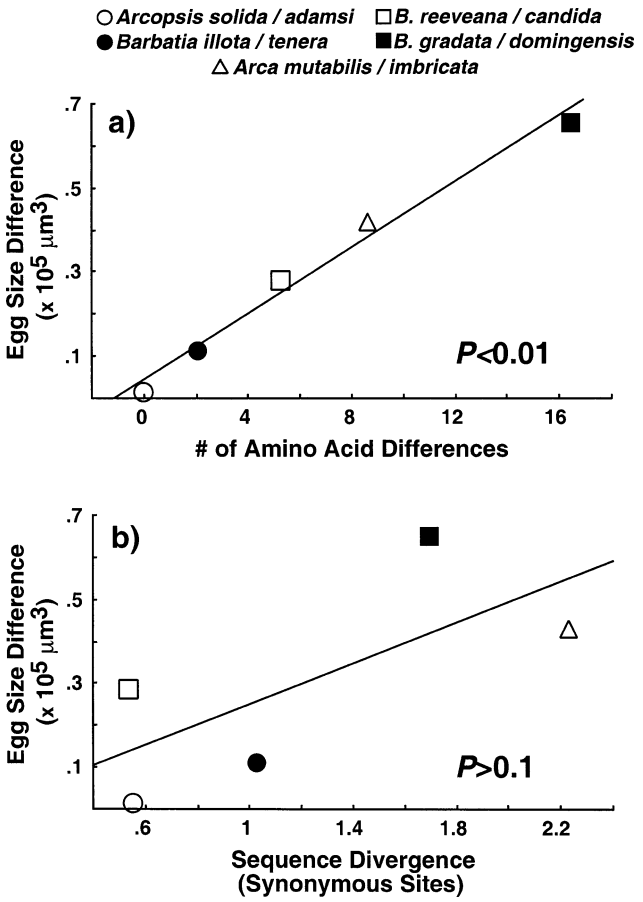

FIG. 2. (a) Correlation between the mean number of amino acid differences and mean difference in egg size among geminate pairs separated by the Isthmus of Panama ($r = 0.988$). (b) Plot of synonymous nucleotide site divergence versus egg size divergence for the same five geminate pairs ($r = 0.724$). *P*-values refer to correlation coefficients.

with a HLRT): synonymous site divergence explains only 52% of the variation in egg size divergence among geminates (Fig. 2b). Substitution of the HKY + G model for models chosen with HLRTs also produced similar results: correlation coefficients were 0.942 ($P < 0.05$) and 0.712 ($P > 0.10$) for the relationships between egg size difference and each of replacement and synonymous site divergences, respectively. Why are amounts of CO1 amino acid differences and egg size divergence so closely correlated? We consider three hypotheses.

First, this correlation is expected if genetic isolation of geminate pairs was staggered through time, and both egg size and CO1 amino acid sequences evolved as clock-like functions of time. Although we cannot currently test predictions about the tempo of egg size evolution, we can examine sequence data for evidence of rate constancy. According to a likelihood ratio test (LRT), synonymous sites evolved in a manner consistent with a Poisson-distributed molecular clock ($2\Delta \ln L = 16.4$, df = 25, $P > 0.90$). Replacement sites, however, whose divergence is correlated with egg size differences, do not satisfy this criterion ($2\Delta \ln L = 44.4$, df = 25, $P < 0.01$). If CO1 replacement site divergence between geminates does not directly reflect relative amounts of time since their isolation, the correlation in Figure 2a cannot be attributed to asynchronous formation of the different geminate pairs combined with constant rates of CO1 and egg size evolution.

A second testable hypothesis is that egg size and CO1 protein evolution are correlated because they are adaptively related, either due to a functional relationship or because the two evolved in response to a similar selective force. Although an adaptive explanation for egg size differences between the Caribbean and the tropical eastern Pacific is likely given the unidirectional changes now documented in both echinoderms (see Lessios 1990) and molluscs, two lines of evidence suggest that patterns of nucleotide substitution in arcid geminates are not consistent with the action of positive natural selection at CO1. First, CO1 amino acid differences across the Isthmus do not appear to represent parallel molecular adaptations to conditions specific to the two oceans. Of 20 amino acid changes in the WA and 25 in the EP, only two changes occurred in more than one EP species and only one occurred in more than one WA species (each amino acid changed was shared by only two species in all three cases). Therefore, it is unlikely that particular amino acid changes are universally favored in either the WA or EP. Tests of selective neutrality (McDonald and Kreitman 1991) also reveal no excesses of replacement substitutions across the Isthmus for each geminate pair (Table 1). Instead, a significant excess of amino acid replacement polymorphism was found within each species pair (Table 1), a pattern consistent with the hypothesis that most replacement site mutations are mildly deleterious (Muller 1964; Ohta 1976, 1992).

Due to an absence of recombination in mtDNA, the particular region of CO1 that we sequenced need not itself be the target of positive selection. Positive selection between oceans combined with complete linkage throughout the

TABLE 1. *G*-tests of independence for selective neutrality (McDonald and Kreitman 1991) between geminate arcid bivalves, with the Williams correction for continuity (Sokal and Rohlf 1981).

Species pair†	Fixed		Polymorphic		*G*	*P*
	Replacement	Synonymous	Replacement	Synonymous		
Arca mutabilis (2)/*A. imbricata* (3)	10	112	5	7	7.51	0.00614
Barbatia reeveana (3)/*B. candida* (2)	3	69	7	3	20.70	0.00001
Barbatia illota (2)/*B. tenera* (4)	1	78	15	4	7.80	0.00521
Barbatia gradata (4)/*B. domingensis* (2)	20	115	13	7	20.50	0.00001
Arcopsis solida (2)/*A. adamsi* (2)§	0	69	0	3	—	—

† Pacific species listed first; samples sizes in parentheses.
§ *G*-test could not be performed for *Arcopsis* because no fixed or polymorphic replacement sites were found.

mtDNA genome, however, should affect CO1 replacement and synonymous divergence in the same way. Given that divergence at synonymous sites is not significantly correlated with egg size divergence (unlike replacement sites), a hypothesis based on the combined effects of selection and hitchhiking also cannot account for the correlation between CO1 replacement site and egg size divergence. In spite of this, error associated with estimates of synonymous sequence divergence is likely larger than the error associated with estimates of replacement site divergence due to multiple substitutions at synonymous sites (Fig. 2; also see Ina 1995). Therefore, a potential correlation between synonymous site divergences and egg sizes could be obscured by the effects of saturation at synonymous sites.

Third, given that egg size differences between oceans are likely adaptive (Lessios 1990) but most CO1 replacement substitutions are not, the close correlation between CO1 amino acid divergence and egg size divergence in arcids (Fig. 2a) may have been driven by a causal factor that simultaneously but independently influenced both adaptive egg size evolution and mtDNA replacement site divergence. The most likely candidate for such a force is historical changes in population size (Omland 1997), because: (1) bottlenecks on population size will increase the fixation rates of weakly deleterious mutations (Muller 1964; Ohta 1976, 1992, 1995; DeSalle and Templeton 1988; Tachida 1991, 1996; Easteal and Collet 1994; Ballard and Kreitman 1995; Nachman et al. 1995; Rand and Kann 1995; Araki and Tachida 1997; Johnson and Seger 2001), and (2) small effective population size (with respect to nuclear genes) combined with high mutation rates and the absence of recombination render the mitochondrial genome particularly susceptible to the accumulation of weakly deleterious substitutions, a process commonly known as Muller's ratchet (Muller 1964; Moran 1996). Likewise, decreases in population size may also increase the response of morphological characters to directional selection (Bryant and Meffert 1995, 1996; Whitlock 1995) in fitness-related traits that otherwise have low heritabilities (Gustafsson 1986; Levin et al. 1991; Price and Schluter 1991; Kruuk et al. 2000), thus increasing the potential for morphological evolution. Therefore, repeated reductions in effective population size in one or both members of a geminate pair could accelerate both morphological and nearly neutral molecular divergence. A related but slightly different possibility is that changing environmental conditions that select for egg size divergence between oceans coincided temporally with historical reductions in effective population sizes that simultaneously drove Muller's ratchet in the mtDNA genome. If either of these mechanisms is responsible for the correlation between egg size divergence and CO1 amino acid sequence, we expect any gene evolving in a nearly neutral fashion to be correlated with egg size divergence between arcid bivalve geminates.

In summary, our results clearly demonstrate that amounts of evolutionary change in molecular sequences and other organismal traits can be tightly correlated even in the absence of an obvious functional relationship or clock-like evolution, and so may reflect common underlying processes. Therefore, the assumption that the evolutionary divergence of molecules and morphology are independent must always be carefully examined. One important implication of these results is that if rates of molecular divergence increase during periods of rapid morphological evolution, molecular-clock based estimates of divergence times among morphologically disparate taxa could be overestimated. For example, estimates of the divergence times of metazoan phyla (e.g., Wray et al. 1996) would be biased if the causes of apparent rapid morphological evolution during the Vendian-Cambrian interval (and later periods of evolutionary diversification) also simultaneously enhanced rates of molecular evolution (Vermeij 1996).

A second implication is that, when properly tested, molecular sequences may reveal much about the tempo and mode of morphological evolution (Pagel 1999). Although the assumption of independence of molecular and morphological evolution is often asserted, comparative methods that use molecular branch lengths to infer ancestral states implicitly assume the existence of a correlation between molecular and morphological characters. In the case of arcid geminates, the existence of a significant correlation between molecular and morphological characters provides an example where reconstruction of ancestral character states with molecular phylogenetic divergences is clearly justified. These data from arcids, therefore, suggest that egg size evolution was not restricted to WA taxa: given that we found a substantial number of amino acid changes in both the WA and EP for most transisthmian pairs, we predict that changes in egg size occurred in both oceans. Therefore, differences between geminate pairs may be due to both egg size decreases and increases in the EP and WA, respectively.

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