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A fossil record of developmental events: variation and evolution in epidermal cell divisions in ostracodes

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SUMMARY The carapaces of some ostracode taxa bear reticulate skeletal ridges that outline underlying epidermal cells. This anatomy allows one to identify homologous cells across individuals, to infer the modal sequence of cell divisions that occurs over ontogeny, and to identify individuals with variant cell patterns (e.g., additional or missing cell divisions), even in fossils. Here we explore the variational properties and evolutionary history of this developmental system in the deep-sea ostracode genus *Poseidonamicus*. Using a sample of over 2000 specimens to capture variation in cell division sequence, we show that phenotypic variation in this system is highly structured: some variants, regions of the carapace, and lineages are much more variable than others. Much of the differences in variation among cells can be attributed to the molt stage in which cells take their final form—cell divisions occurring later in ontogeny are more variable than those earlier. Despite ample variation, only two evolutionary changes in the sequence of cell divisions occur over the 40 Myr history of this clade. The evolutionary changes that do occur parallel the two most common intraspecific variants, suggesting that developmental structuring of variation can have long-term evolutionary consequences. Analysis of the most common variant over the last two molt stages suggests that it suffers a fitness disadvantage relative to the modal form. Such normalizing selection may contribute to the evolutionary conservativeness of this developmental system in the Ostracoda.

INTRODUCTION

All evolutionary processes require the heritable variation ultimately supplied by mutation. Although mutations are random with respect to the present needs of an organism (Lenski and Mittler 1993; Brisson 2003), their effects are mediated by development and thereby nonrandomly expressed in phenotypes (Brakefield 2006). One consequence of this developmental structuring is that different phenotypic variants are generated with greatly differing frequencies. Assessing the importance of structured phenotypic variation in channeling or constraining evolutionary trajectories is a major goal of evolutionary biology (Maynard Smith et al. 1985; Arnold 1992; Shubin et al. 1995; Schluter 1996; Arthur 2002; Brakefield 2006; Hendrikse et al. 2007).

Although paleontological data can contribute meaningfully to the ongoing synthesis of evolution and developmental biology (Raff 2007), the morphology preserved in fossils does not often allow access to developmental events and processes. Sometimes, developmental patterns in extant relatives can guide inferences for fossil taxa (Valentine 2006; Erwin 2009), and patterns of relative growth and variability can be used to infer aspects of the underlying developmental processes (Fusco et al. 2004; Webster and Zelditch 2008). Only rarely does the morphology of fossils preserve more direct imprints of specific developmental processes, such as larval mode in some invertebrates (Jablonski and Lutz 1983; Jeffery and Emlet 2003), spore formation in lycopods (Looy et al. 2005), or pelvis reduction in stickleback fish (Bell et al. 2006). Although all morphological structures potentially inform us about their generative developmental processes, these examples are unusual in that specific variations can be linked to alternative outcomes of identifiable developmental processes or events.

In this article, we describe and exploit another exceptionally informative anatomical system: the cell-reflecting skeletal mesh characteristic of the shells of many ostracode taxa. The reticulate pattern of ridges on the valves of these ostracodes traces the outlines of underlying epidermal cells (Okada 1981, 1982b), a remarkable convenience that permits the identification of homologous cells and sequences of cell divisions, even from fossil specimens. We focus on the genus *Poseidonamicus*, and use its rich fossil record to explore the evolution of this developmental system. We proceed in three main sections. First, we describe the cell-reflecting morphology of *Poseidonamicus* and indicate how we recognized specific cell divisions and documented variant outcomes of these events. Next, we assess the degree to which variation in this developmental system is structured such that certain developmental events, regions of the carapace, lineages, environments, or time

intervals are more prone to vary than others. Finally, we assess the evolutionary consequence of this variation by estimating the relative fitness of variant morphologies, and by evaluating the potential for developmental processes to constrain or channel evolutionary changes in this clade.

THE DEVELOPMENTAL SYSTEM

Homologous cells

Carapaces in many ostracode taxa bear a reticulate mesh of ridges that outline excavate compartments called fossae. When fossae are regular and relatively large, this condition is called macroreticulation (Liebau 1991). With detailed anatomical work on several living macroreticulate species from two different families, Okada (1981, 1982a, b) demonstrated that each fossa on the carapace corresponds in a one-to-one manner to an underlying epidermal cell (see also Keyser 1995).

Unlike other fossil examples in which cells of multicellular organisms can be discerned (e.g., Conway Morris and Harper 1988; Masterson 1994; Organ et al. 2007), fossae in many ostracode carapaces can be individually homologized across specimens and taxa (Benson 1971, 1972; Liebau 1971; Irizuki 1994, 1996; Schornikov and Tsavera 2002). It is possible to recognize specific fossae because they are generally consistent in number and arrangement within species (Benson 1972; Okada 1982b; Abe 1983; Liebau 1991), because they have characteristic shapes and features, and because other anatomical landmarks such as pores, ridges, and muscle scars can be used as reference points.

In the present study, we focused efforts on fossae located in the postero-central region of the carapace of *Poseidonamicus*, bounded anteriorly by the adductor muscle scars, ventrally by the prominent ventral ridge, dorsally by the dorsal ridge, and posteriorly by the vertical ridge connecting the termini of the dorsal and ventral ridges (Fig. 1). This region of the carapace was singled out for close analysis because the wealth of anatomical features in that region (major ridges, muscle scars, pores) facilitates the identification of specific fossae (see Hunt 2007b). To keep track of individual fossae, we used a labeling scheme with approximately vertical columns denoted by a letter; fossae within columns were numbered from ventral to dorsal (Fig. 1). The column bordering the adductor muscle scars is called the M-column, with subsequent columns labeled A through D from anterior to posterior. The row and column designations reflect fossae positions, but otherwise do not imply developmental commonality.

Cell divisions through ontogeny

In reticulate species, the number of fossae increases from one juvenile instar (= molt stage) to the next. (By convention, the adult stage in ostracodes is denoted A, and instars are counted backwards from there: A-1, A-2, and so on.) Close examination can reveal which fossae and cells have divided at each molt because one larger fossa will usually be replaced in the subsequent instar by two smaller fossae in the same position. On this basis, workers have inferred sequences of epidermal cell divisions in several different taxa (Okada 1981; Liebau 1991; Hunt 2007b). Because the reticulation pattern for the new instar is already present at ecdysis (Okada 1982a), the epidermal cells must divide sometime before molting initiates.

The ontogenetic progression of fossae in *Poseidonamicus riograndensis* is shown in Fig. 2 with the sequence of cell divisions hypothesized by Hunt (2007b) indicated. The primary evidence for inferring specific cell divisions is the matching of size and shape from daughter cells to a parent cell in the previous instar. For example, two large and broad fossae, labeled A1B1 and A2B2, are located in the center-ventral part of this region in instar A-2. In the subsequent instar, A-1, these fossae are each replaced by pairs of fossae that together form similar shapes (Fig. 2). This inference is supported by the conserved location in both instars of homologous pores on the ridge between the B and C columns, and on the ridge between M and A columns (Fig. 2). The added fossae in this region insert in between these vertical ridges, indicating that the cell divisions occurred in the A1B1 and A2B2 fossae, and not in those from the C-column. Similar reasoning was used for the remaining inferred cell divisions (Liebau 1991; Hunt

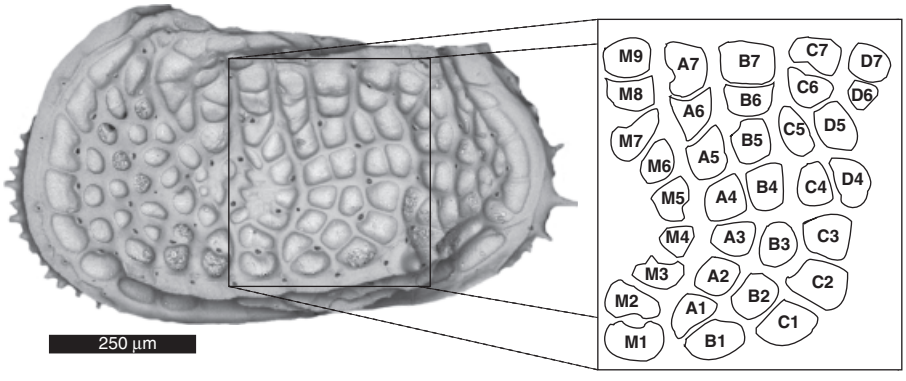

Fig. 1. Left valve of an adult *Poseidonamicus pintoi* from the Quaternary of the North Atlantic Ocean (USNM 527093), showing labeling scheme for skeletal compartments called fossae. Anatomical studies of related, living ostracodes suggest that each fossa corresponds to an underlying epidermal cell. Anterior is to the left, and dorsal is up.

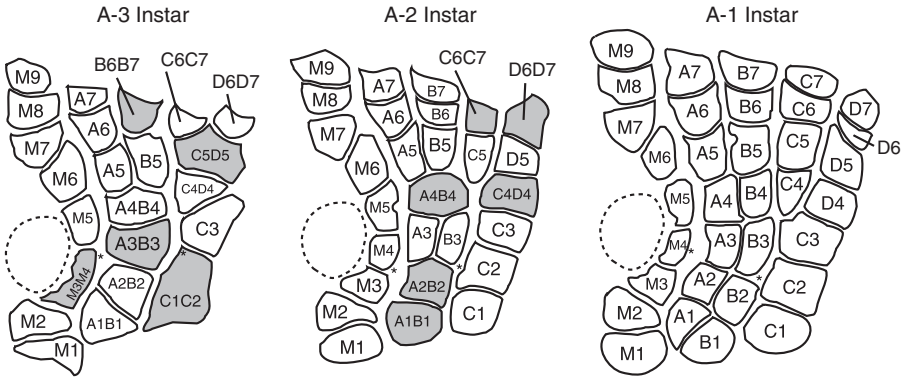

Fig. 2. Ontogenetic sequence of fossae arrangement through three molt stages (instars), proceeding from left to right. The number of fossae increases with each instar resulting from cell divisions in the epidermal cells underlying the fossae. Those filled gray are inferred to divide before the next instar according to the scheme of Hunt (2007b). Fossae are labeled with a letter indicating their vertical column, and a number indicating ventral-to-dorsal position in the column. Fossae in earlier instar stages are labeled according to their inferred daughter fossae in the final, A-1, configuration. Specimens figures are *Poseidonamicus riograndensis* from the Miocene, but except for the two evolutionary changes described in the text, this appears to be the modal pattern for all *Poseidonamicus* species. Shown are left valves; anterior is to the left of the figure. SEM images of these specimens are given in Fig. 3 of Hunt (2007b). Dotted line outlines area of attachment for adductor muscle scars, and the asterisks note the position of two homologous pores discussed in the text.

2007b). In total, five cells are inferred to divide between the A-3 and A-2 instars, and six divide between instars A-2 and A-1 (Fig. 2). The arrangement of fossae is exactly the same in the adult and last juvenile (A-1) instars, a pattern that has been observed in other reticulate ostracode taxa (Okada 1981; Liebau 1991). With just a few exceptions that will be discussed below, this sequence of cell divisions is conserved through the 40-Myr history of *Poseidonamicus*.

Variant cell patterns

Most specimens of *Poseidonamicus* conform to the fossae configuration that is typical for their species. However, some individuals show clearly variant patterns, and in many cases these variants can be ascribed to specific deviations in the sequence of cell divisions. For example, the most common variant encountered in *Poseidonamicus* bears two fossae in the area in which the C3 fossa usually resides. This extra fossa implies that an excess cell division has occurred relative to the typical ontogenetic sequence, and the spatial position of these fossae is consistent with the extra split occurring in the epidermal cell underlying the C3 fossa (Fig. 3, A and C; see Hunt 2007b). This particular variant is always accompanied by the absence of the normally occurring pore located near the antero-dorsal corner of the C3 fossa (Fig. 3D; see Hunt 2007b). Another kind of variant occurs when a specimen has one large fossa in an area usually occupied by two smaller fossae. If this large fossa is inferred to be parental to the two smaller ones in the typical ontogenetic sequence, then the observed variant likely results from the failure of a usual cell division to occur (Fig. 3D). Other cellular variants have the typical number of fossae in a region, but differ in their arrangement. One version of this pattern occurs when daughter fossae from the same parental cell are present, but positioned orthogonally to their normal relationship. This pattern is consistent with the parental cell dividing along a different axis than is typical (Fig. 3B).

We systematically documented variation in fossae arrangement in 2193 specimens of deep-sea *Poseidonamicus* from adult ($N = 869$) and A-1 ($N = 1324$) instars. This total includes all specimens preserved well enough to allow fossae patterns to be observed, and it includes left and right valves (deep-sea ostracode fossils are rarely found as articulated carapaces). These specimens span the phylogenetic, geographic, and temporal scope of this genus; localities and ages of analyzed samples are detailed in Hunt (2007a, b). For each specimen, we identified fossae variants and scored them using a two-part code. The first part of the code indicates the fossa or fossae involved, and the second part indicates the nature of the interpreted cellular variation. Five categories of variant were employed: *split* (extra fossa), *unsplit* (missing fossa), *direction* (division occurs roughly orthogonal to modal pattern), *position* (typical complement of fossae are present, but with substantial alteration of relative positions), and *aberrant* (other variations). The last is a catch-all category for specimens with anomalous fossae arrangements that cannot easily be interpreted in terms of specific deviations to the sequence of cell divisions. Multiple fossa may be implicated in a particular variation, and multiple variants could be observed from the same specimen (Fig. 3C). As an example, the presence of an extra fossa interpreted as a cell division of the C3 fossa was recorded as *C3-split*; other example variants and their codes are provided in Fig. 3. Each specimen was considered variant or not by reference to the modal sequence for its species, so that the few evolutionary changes in this system did not confound measurements of within-species variation.

Uncertainty in coding was accommodated at several levels. If there was doubt as to whether an individual displayed a variant pattern relative to its species' modal configuration, a question mark was added to the code. All the analyses

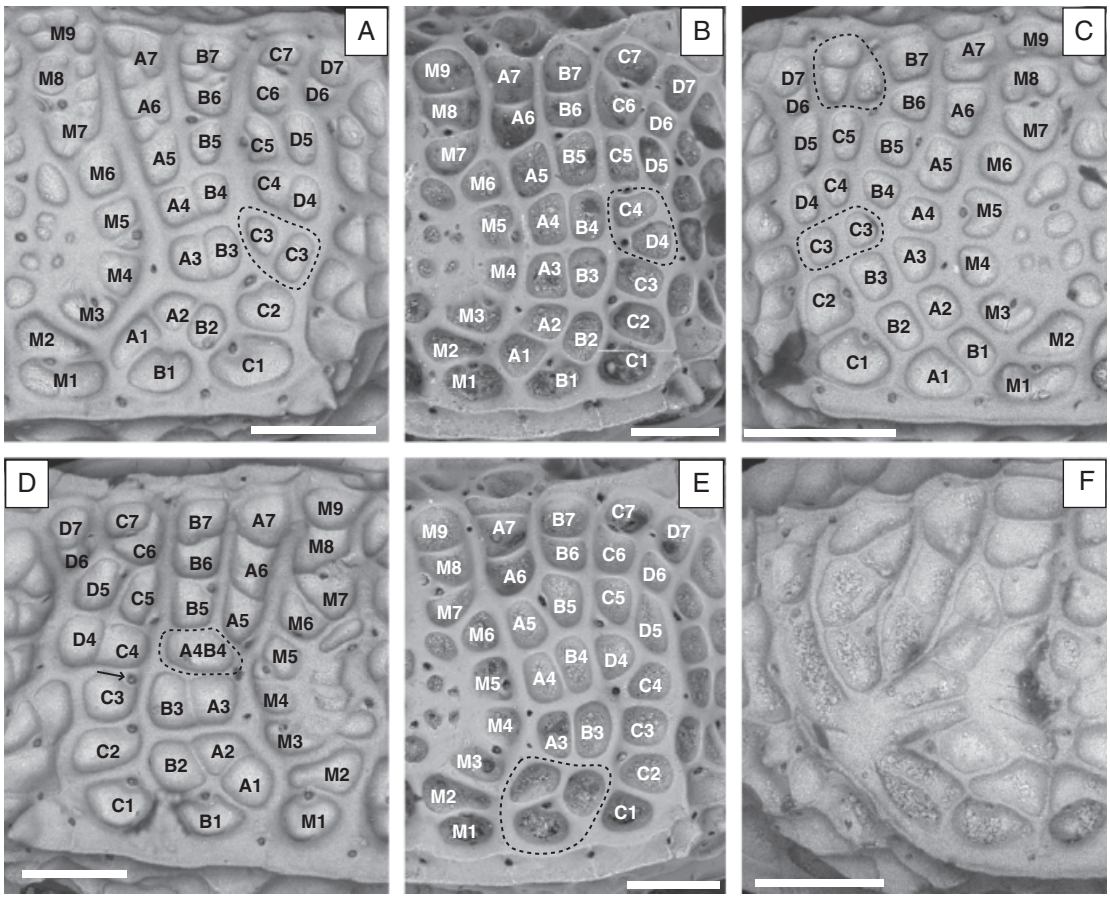

Fig. 3. Examples of *Poseidonamicus* specimens bearing variant fossae arrangements. (A) Left valve of *Poseidonamicus pintoi* with an extra cell division in the C3 fossa, coded as *C3-split*. (B) Left valve *P. pintoi* specimen with variant direction of splitting for the C4/D4 fossae, *C4/D4-direction*. (C) Right valve of *P.* species 4 bearing two variations: *C3-split* and *C6/C7-split*. (D) Right valve of *P. pintoi* lacking a normally occurring cell division, *A4/B4-unsplit*. (E) Left valve of *P. pintoi* lacking a typical cell division in the ventral region of the A and B columns, *A1/B1/A2/B2-unsplit*. (F) Right valve of *P. miocenicus* with widespread disruption throughout the focal region, perhaps as a result of injury. Arrow in (D) indicates pore that is absent with the *C3-split* condition. Scale bars = 100μm; dotted lines highlight fossae interpreted to differ from the modal pattern. All individuals are adults, except for (A) and (C), which are from the last juvenile molt stage (A-1 instar).

reported here were repeated included and excluding these questionable variants. Since none of the results differed appreciably across the two treatments, only the results that included questionably variant individuals are reported. Often, a specimen would clearly differ from the typical pattern, but uncertainty remained about exactly which fossae were involved. For example, the specimen in Figure 3C has an extra fossa in the vicinity of fossae C6 and C7, but it is not clear which is responsible for the extra cell division. In these cases, the variant would be spread across both fossae (*C6/C7-split*), and in the statistical compilation, each fossa would be credited with half of a variant. More generally, if the uncertainty included n fossae, each possibly involved fossa is credited with $1/n$ of a variant. A very small number of individuals showed very widespread fossae disruption, possibly the result of an injury incurred during molting (Fig. 3F).

Several factors complicate accurate scoring of variation. Secondary reticulation can overlay the cell-reflecting primary mesh (Okada 1982b; Fitz-Gerald 1983; Kamiya 1992). In *Poseidonamicus*, secondary reticulation usually decreases through ontogeny, so this effect is most operative in juvenile (A-1) individuals, and in species of the clade including *P. dinglei*, *P. anteropunctatus*, and *P. nudus*, which are characterized by a reduction in the height of the primary reticulation. Careful study can usually distinguish primary from secondary reticulation because the latter is lower and more irregular than the former, and judgments of split fossae were only employed if secondary reticulation could be ruled out. However, the reticulum of *P. praenudus* and *P. nudus* is so reduced as to prevent accurate scoring in these taxa, and they were excluded from this analysis. In more robust taxa, some ridges are very prominent, whereas others can be much

reduced, and it is possible to confuse a very reduced ridge separating two fossae with a missing cell division. Often, careful examination reveals the trace of a ridge, especially at the margins of the fossa. We were cautious about assigning *unsplit* variants in these situations, only doing so when it appeared unlikely that the putatively absent ridge was just very reduced. It is helpful that secondary reticulation and ridge reduction both affect entire regions of the carapace, rather than individual fossae. As a result, in evaluating interpretations of reduced ridges or secondary reticulation, the morphology of neighboring fossae can be used as a guide.

A final complication concerns the coding of relative minor variations in fossae positions. The *position* variant category was designed to capture large, discrete variants and not minor quantitative variation in fossae locations. However, positional variation ranges from trivial to dramatic, with no obvious threshold separating major from minor variation. Our approach was pragmatic in that we attempted to score all variants for which the difference from the modal pattern was large enough to be recognized and categorized, although this practice will necessarily entail some subjectivity. Note this only applies to the *position* category (and to a lesser extent to the *aberrant* and *direction* classes); *split* and *unsplit* are truly discrete conditions.

For maximal consistency, the first author scored all specimens after reviewing each specimen at least three times. In order to assess the repeatability of this procedure, the second author independently scored a subset of 75 specimens, and the two sets of scores were compared. Finally, we note that although our categories imply a process interpretation, even if these processes are wrongly inferred, the codes still represent repeatable and meaningful phenotypic variation in developmental outcomes.

VARIATION: MAGNITUDE AND STRUCTURE

Frequency of variants

Of the 2193 individuals scored, 303 (13.8%) were judged to have at least one variation present in the postero-central region of the valve. Where variants could be unambiguously interpreted, they appeared at vastly different frequencies. By far the most commonly occurring variant was the presence of an extra cell resulting from a cell division in the C3 fossa (Fig. 4). Next most frequent was an extra cell division in the C6/C7 fossae, along with variation in the direction and occurrence of the cell division leading to the C4 and D4 fossae (Fig. 4). Most variant individuals bore a single detectable variation (219/303), but some individuals were interpreted to bear multiple separate variant fossae conditions. These individuals bearing multiple variations were more numerous than expected if variants were independent ($\chi^2 = 121.6$, df = 2, $P = 2.2e{-}16$; comparing the number of individuals with 0, 1, and >1 variants to the Poisson expectations). In total, there were 0.185 variants observed per specimen, 0.137 if the highly variable C3 fossa is excluded.

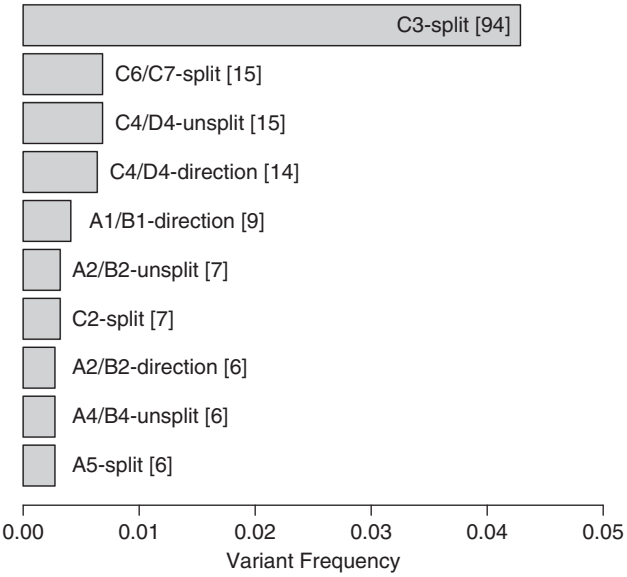

Fig. 4. Frequencies of the 10 most common developmental variants. Numbers in brackets indicate counts of individuals bearing each indicated variant pattern. Only variants that could be interpreted confidently were included in this tabulation.

Spatial and ontogenetic patterns

The anatomical distribution of fossae variation is far from uniform. Figure 5 shows a map of the studied fossae, with the darkness of each fossa indicating its frequency of variation. Fossa C3 was excluded from this comparison because of its greatly elevated variation would dominate, masking any other structure. Even omitting C3, this figure reveals a great range of realized variability across fossae. The most consistent fossa is M6 with 0.00058 variants per specimen. Only three specimens were inferred to vary at M6, and two of these showed disruption through a large region affecting many fossae, and thus may reflect injuries rather than specific developmental variation. The most variable fossa is C3 (0.048 variants per individual), followed by its neighbor C4, which accrued 0.011 variants per individual scored. Overall, frequencies of variation are very unevenly distributed across fossae—the rate of variation of the most variable fossa (C3) is over 80 times greater than that of the most constant fossa (M6).

The variation map (Fig. 5) hints at some spatial regularities. The least variable fossae are in the anterior M-column, and the most variable are in the posterior C and D columns. There also appears to be a trend of decreasing variation from ventral to dorsal, at least within the M, A, and B columns. Caution is advised, however, because these relationships may be confounded by the order in which cells divide. The

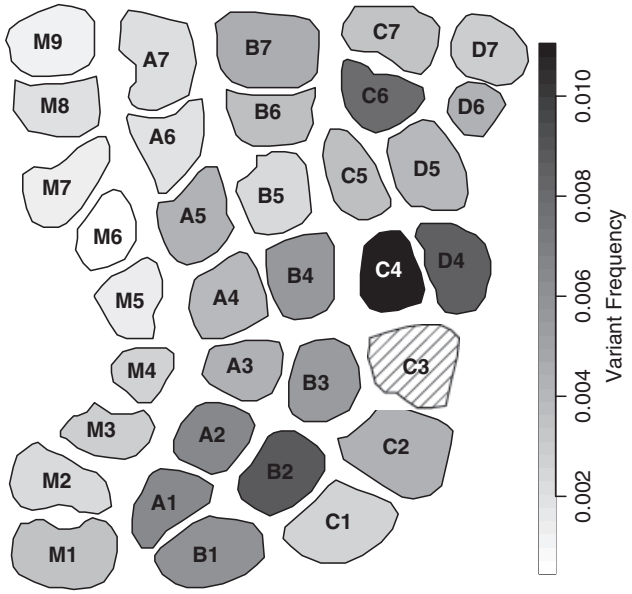

Fig. 5. Variation map showing the frequency of variation for all fossae in the analyzed region of the carapace. Darker fills indicate higher levels of variation. Because it is so much more variable than the other fossae, C3 was omitted in order to allow patterns among other fossae to be discerned. Left valve shown; anterior is to the left.

low-variation M-fossae finalize early in development, and several of the most variable fossae are among the latest to form (e.g., C6, C4/D4; see Fig. 2). The earlier finalization of the M-fossae relative to those more posterior may relate to the general pattern in which limbs and other structures develop from anterior to posterior in ostracode ontogeny (e.g., Okada et al. 2008).

We used multiple regression to test how well fossa variation could be predicted by anatomical position and ontogenetic order. The dependent variable in all analyses was the per-individual frequency of variants for all fossae. For these and all subsequent regressions, these frequencies (f) were arcsine transformed, $\sin^{-1}(\sqrt{f})$, which is a standard technique for stabilizing the variance and improving the normality of proportions (Sokal and Rohlf 1995). The exact position of each fossa varies across species and specimens, and so the row and column designations were used as proxies for location. Antero-posterior (AP) position was taken as the integer order of columns ($M = 1$, $A = 2$, $B = 3$, $C = 4$, $D = 5$), and dorso-ventral (DV) position was taken as the row number of the fossa, divided by the total number of fossae in the column (this division renders comparable columns with differing numbers of fossae). The ontogenetic stage (ONTO) of each fossa was scored as the instar in which it takes its final form according to Fig. 2 (ONTO values: A-3 or earlier = 1, A-2 = 2, A-1 = 3). The C3 fossa was omitted because, if included, it always is an extreme outlier with very high leverage. Moreover, as *C3-split* is an order of magnitude more common than most other variants (Fig. 4), it seems unlikely that its incidence is governed by any morphological or ontogenetic gradient one may hope to uncover through these regressions.

Table 1. Results of the regressions predicting fossae variation by with antero-posterior position (AP), dorsal-ventral position (DV), and the ontogenetic stage at which each fossa finalizes (ONTO)

	Model	R^2	AIC_C	Akaike weight	Significant terms
1.	Intercept only	—	− 166.72	0.000	—
2.	AP	0.25	− 175.31	0.000	AP
3.	DV	0.10	− 167.36	0.000	—
4.	AP+DV	0.43	− 181.33	0.001	AP, DV
5.	ONTO	0.58	− 193.89	0.328	ONTO
6.	ONTO+AP	0.58	− 191.68	0.109	ONTO
7.	ONTO+DV	0.62	− 193.97	0.341	ONTO
8.	ONTO+AP+DV	0.63	− 193.10	0.221	ONTO

Given are the proportion of variance accounted for (R^2), the small-sample Akaike Information Criterion (AIC_C), Akaike weights and the model terms that are individually significant ($P < 0.05$). The C3 fossa was omitted for reasons explained in the text.

The eight models with all possible configurations of the three independent variables are listed in Table 1, along with measures of model success. Akaike Information Criterion scores (AIC_C, to indicate the small-sample version; Anderson et al. 2000) indicate that there is substantial structure in fossae variation with respect to anatomical position, ontogenetic formation, or both. All models that receive more than a trivial amount of Akaike weight include the ontogenetic term (ONTO), and in all of these models this term—and this term alone—is significant (Table 1; DV is also nearly significant in model #8, $P = 0.056$). The significant relationship between variation and AP position in model #2 seems to arise because of the anterior-posterior structure in the timing of fossae development. But the effect of AP disappears in all models that also include ONTO, suggesting ontogenetic timing is the primary driver of these spatial patterns (Table 1).

Although these comparisons establish ONTO as more important than the spatial variables for governing fossae variability, still there are several models (#5–8) that receive nontrivial model support. In such cases, it is advisable to consider parameter estimates averaged over all models, proportional to the support that each model receives (Anderson et al. 2000). These model-averaged estimates (with standard errors in parentheses) are as follows: AP, 0.0007 (0.0014); DV, − 0.007 (0.09); ONTO, 0.016 (0.003); these calculations were performed using the R package MuMIn (Bartoń 2009). Only the coefficient for the ontogenetic term is significantly different from zero, once we account for the uncertainty in determining the best-supported model. Its coefficient is positive, indicating that later-forming fossae are, on average, more variable than those established earlier in ontogeny.

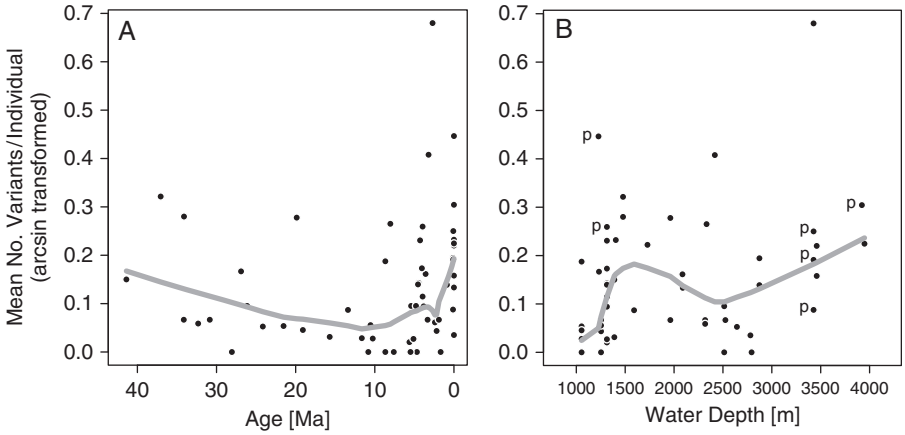

Fig. 6. Plot of variant frequency by sample with respect to (A) geological age, in millions of years ago, and (B) water depth. Gray lines are locally weighed (lowess) regressions using a moving window that spans 50% of the data. Only samples with at least 15 individuals are plotted; populations assigned to the relatively variable species *Poseidonamicus pintoi* are labeled as "p" in (B). Variation at the C3 fossae was not included in these plots or analyses; variant frequency arcsin transformed as described in the text.

Temporal and bathymetric patterns

Little systematic relationship between variation and geological age is apparent (Fig. 6A). Total variation across samples increases with sample depth, but this largely is driven by the lack of samples with very little variation in very deep (>3000 m) samples (Fig. 6B), and in any case this model explains little of the variance in variation levels across samples (Table 2). Moreover, differences among species and clades may overprint on these results. For example, *Poseidonamicus pintoi* is common at Quaternary deep-water sites, where it generally shows high variation. However, the two relatively shallow samples of *P. pintoi* are also highly variable, suggesting that abundant variation may be a property of this taxon and not necessarily its deep-water environment (Fig. 6B). Species are too consistent in their depth of occurrence to be able to tease apart these kinds of phylogenetic effects more systematically.

Right-left repeatability of variants

If variations have a genetic basis, they will generally occur on the left and right sides of bilaterally symmetric organisms. Alternatively, many variations that reflect nonheritable noise will be expressed on only one side (fluctuating asymmetry) (Møller and Swaddle 1997) and the left-right repeatability of a variant provides an upper bound on its heritability (Falconer and Mackay 1996). Because articulated carapaces of *Poseidonamicus* are rarely encountered, it is impractical to perform a large-scale analysis of right-left repeatability of fossae variation. However, by searching through the collections of the National Museum of Natural History, we located five articulated carapaces that bear the *C3-split* variant. In four of these individuals the *C3-split* variant was clearly present in both left and right valves. This high left-right repeatability is consistent with the *C3-split* variant having some genetic basis.

Table 2. Regression of the variation of populations against their geological ages and bathymetries

	Model	R^2	AIC_C	Akaike weight	Significant terms
1.	Intercept only	—	−16.92	0.030	—
2.	Depth	0.14	−23.30	0.718	Depth
3.	Age	0.01	−15.29	0.013	—
4.	Depth+Age	0.14	−21.10	0.239	Depth

The 50 populations with sample sizes ≥15 were included. Variants of the C3 were omitted for reasons indicated in the text.

Repeatability of scoring

In order to assess the repeatability of variant scoring across individuals, the second author independently scored a sample of 75 individuals from this dataset. In this sample, specimens with some kind of variant according to GH were over-represented (51/75), as were *C3-split* individuals (23/75), but were otherwise chosen at random. Although both scorers used as a reference the sequence of cell divisions outlined above, they otherwise worked completely separately, without any consultation or discussion about strategies until after the scoring had been completed.

The concordance between scorers can be assessed at different levels of specificity. There was very good correspondence in identifying variant versus nonvariant individuals. Of the 24 specimens scored as normal by GH, 22 were scored the same way by MY. The two specimens that differed were interpreted by GH to have reduced ridges, but by MY to have possibly unsplit fossae. In addition, both scorers generally agreed when the C3 fossa was split: of the 23 individuals scored as unambiguously *C3-split* by GH, 19 were so scored by MY. Of the four specimens scored differently, MY agreed there was an extra cell division, but assigned it to a different fossa. Part of the discrepancy was because GH but not MY took the absence of the antero-dorsal pore near C3 as evidence for the

C3-split condition because these two variations are associated (Hunt 2007b).

Most codings for rarer variants were congruent, but not always exactly the same between scorers. Of the 65 variations identified by GH, MY coded 40 as either exactly the same, or compatible but with a different level of specificity (e.g., *C3-split* vs. *C3/C4-split*). Six differences involved conflicting interpretations about secondary reticulation or reduced ridges, and three involved clear errors by one of the scorers. Twelve variations were identified as the same category across scorers (e.g., *split*), but were assigned to nearby but different fossae. Finally, the four remaining variations represented plainly dissimilar interpretations.

EVOLUTIONARY DYNAMICS

Evolutionary transitions in fossae arrangement

Despite the abundance of within-species phenotypic variation, only two qualitative changes in fossae arrangement have occurred in the over 40 Myr history of *Poseidonamicus* (Fig. 7). Both changes involve fixing an extra cell division in a lineage, and both occur early in the history of the clade. One change results in the fixation of the *C3-split* variant in the lineage leading to the clade consisting of *P. dinglei*, *P. anteropunctatus*, *P. punctatus*, *P. praenudus*, and *P. nudus* (see fig. 9 in Hunt 2007b). Although the reticulum is much reduced in *P. nudus*, careful observation under scanning electron microscopy and transmitted light microscopy (Benson 1972) confirms that this species bears a divided C3 fossa, and we have never observed any individual of this clade with a reversion to the unsplit C3 condition. The second apomorphic change in fossae arrangement also occurs within this clade, within a lineage of *P. dinglei* from the central Pacific Ocean (Deep-sea Drilling Program site 463, 31.9–30.5 Ma). These specimens bear an extra fossa in the C6/C7 region, a condition that is only present rarely in *P. dinglei* from other localities. This species persists at DSDP 463 into the Miocene (Boomer 1999), but it is unclear if the split C6/C7 condition is dominant through this whole interval.

Note that both apomorphic conditions (*C3-split*, *C6/C7-split*) appear elsewhere in the genus as within-population variants. In fact, they are the two most common variants across the genus out of >70 specified kinds of variant (Fig. 4). There are additional, smaller evolutionary changes in fossae position in *Poseidonamicus*, some of which have been used to inform phylogenetic inference (Hunt 2007b). However, these reflect relatively minor changes in position, under the threshold of major qualitative variation scored here.

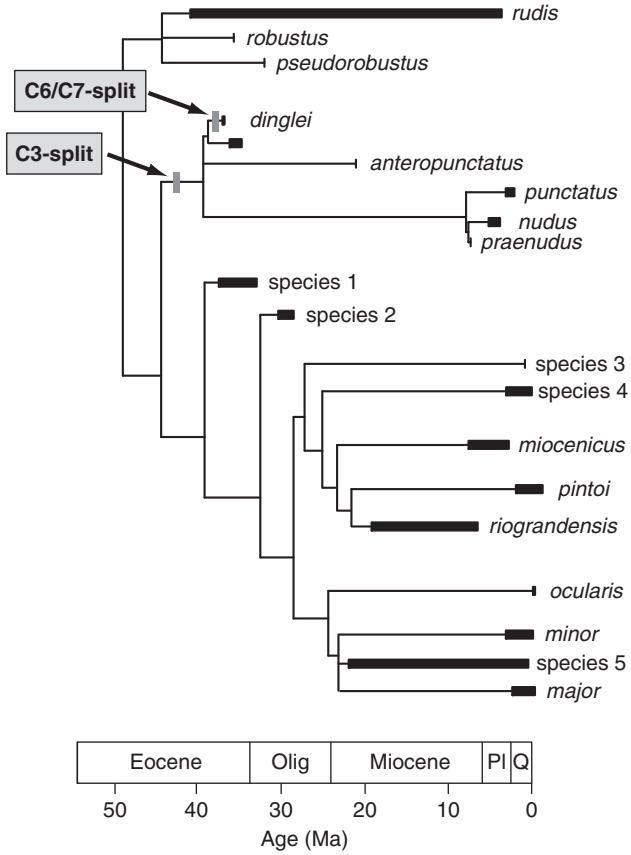

Fig. 7. Phylogeny of deep-sea *Poseidonamicus*. Topology and stratigraphic ranges are from Hunt (2007b). The two known qualitative evolutionary changes in fossae arrangement are indicated with gray bars, denoting the fixation of the split C3 condition at the base of the clade that includes *P. dinglei* and *P. nudus*, and fixation of extra fossae in the vicinity of C6 and C7 in one regional lineage of *P. dinglei*. Bottom scale shows the geological time scale with Cenozoic epochs and ages in millions of years before the present.

Natural selection and fossae variants

Because the adult and A-1 instars share the same arrangement of fossae, the frequencies of specific variants in two ontogenetic stages can be compared. Such frequencies are often used to measure natural selection in wild populations: all else equal, the variant with greater fitness should increase in dominance over ontogeny because of its greater survivorship. Here we use the variant frequencies in these two instars to compute the relative fitness of the most abundant variant, *C3-split*.

The log-likelihood function for relative fitness of two morphs, given frequencies before and after selection is given by Kalinowski and Taper (2005). In this context, before selection is the A-1 instar and the adult frequencies are those after selection. Thus, we can measure net selection during the interval bounded by the A-1 and adult molts. The maximum-likelihood estimator of fitness for a variant morph is $w_V = (f_{A,V}\, f_{A\text{-}1,M})/(f_{A,M}\, f_{A\text{-}1,V})$, where f indicates the frequencies, with a first subscript indicating ontogenetic stage (adult or A-1), and the second subscript referring to the variant (V)

Table 3. Frequencies of occurrence of the *C3-split* variant, partitioned by instar and species

	A-1 Instar		Adult instar			
Species	*C3-split*	*C3-whole*	*C3-split*	*C3-whole*	Proportion *C3-split* (A-1)	Relative fitness (*C3-split*)
P. major	2	203	0	96	0.01	0.00
P. minor	0	46	0	17	0.00	—
P. miocenicus	13	324	8	206	0.04	0.97
P. pintoi	13	180	10	144	0.07	0.96
P. riograndensis	5	156	2	108	0.03	0.58
P. rudis	0	63	0	24	0.00	—
Species 1	3	27	1	27	0.10	0.35
Species 3	5	45	0	43	0.10	0.00
Species 4	10	84	5	58	0.11	0.73
					Across all species	0.69
					95% CI	[0.46, 0.99]

These frequencies allow calculation of fitness of the *C3-split* variant, relative to the modal (unsplit) morphology. All fitness estimates are less than unity, but only in *Poseidonamicus* species 3 is this difference significant (i.e., the 95% confidence interval excludes 1.0). Pooled across species, the fitness estimate is 0.69, and its confidence limits barely exclude one. Species represented by at least 50 scored individuals were included, except for the five species for which a split C3 fossa is the fixed condition (see Fig. 7). When the proportion of A-1 juveniles with the *C3-split* condition is zero, fitness cannot be calculated and these taxa therefore do not contribute to the estimation.

or modal (M) morph. For all species represented by at least 50 scored individuals pooled across samples, we measured the fitness of the *C3-split* versus the modal morph (Table 3). In order to increase sensitivity, we also estimated relative fitness jointly across species while allowing each species to have a different frequency of incidence for *C3-split*. We used Kalinowski and Taper's log-likelihood function to compute profile confidence intervals for relative fitness as the range of values of w_V that yield log-likelihoods within 1.92 values of the maximum (Pawitan 2000).

The incidence of the *C3-split* morph varies across taxa (Table 3). For two of the species, *P. minor* and *P. rudis*, no variant individuals were found, but the incidence ranged upwards to 10% (Table 3). In all nine species, the proportion of *C3-split* individuals in the adult instar is lower than or equal to that of the A-1 stage. This pattern is consistent with *C3-split* incurring a fitness cost relative to the modal morph. The fitness difference is individually significant in only one species (*P.* species 3), but the joint fitness estimate across all species, $w = 0.69$, indicates that these variants incur a substantial reduction in fitness. The confidence interval on this estimate is quite broad (Table 3), though it does barely exclude the possibility that the variant and modal morphs are equally fit ($w = 1$).

This analysis makes a few important assumptions. For the joint fitness estimate, natural selection is assumed not to differ across species, sites, or time intervals. Heterogeneity in these conditions produces a fitness estimate that is essentially a weighted average across these factors. In addition, sampling error in estimating proportions in the A-1 instar is ignored (Kalinowski and Taper 2005, p. 694). It is also assumed that no conversions among the morphs occur after the A-1 instar, that is, undivided C3 fossae do not subsequently split nor do already split C3 fossae subsequently remerge. If the latter occurs (the ostracode "corrects" the variant) this would decrease the incidence of the *C3-split* in adults without natural selection. It does not seem developmentally plausible that daughter cells merge after dividing, and more complicated scenarios involving cell migration or apoptosis are unlikely because the epidermal cells are anchored in place (Okada 1982a). It is harder to rule out the possibility that C3 fossae that are undivided in A-1 subsequently divide before the adult instar. If this does occur, however, it would add to the frequency of *C3-split* adult variants, and thus imply that the fitness cost to the C3 variant is even greater than suggested by Table 3.

DISCUSSION

Ample variation, little evolution

The conservativeness of fossae arrangement is striking, especially given the documentation of high levels of standing variation. With more than 10% individuals bearing some kind of deviation from modal patterns, why are evolutionary differences in fossae arrangement so uncommon? In fact, the evolutionary stability may be even more extensive than documented here, encompassing related genera such as *Bradleya*. Liebau (1991) reconstructs epidermal cell divisions in the ontogeny of *Bradleya praemckenziei*, and his inferred divisions are entirely consistent with those inferred in *Poseidonamicus* (Fig. 2; but note that Liebau does not try to interpret the divisions in the C6/C7/D6/D7 region). This correspondence is remarkable because the *Poseidonamicus* sequence was inferred

without knowledge of Liebau's *B. praemckenziei* sequence, and it suggests that subjectivity in inferring fossae does not obscure underlying biological patterns, as does the general repeatability of scoring among the authors in the present study.

Several nonexclusive explanations may account for the presence of abundant phenotypic variation in the face of widespread evolutionary conservation. First, the variation may not be heritable, and therefore unavailable to evolutionary processes such as selection and drift. Second, individuals bearing variant morphologies may incur a fitness cost.

The observation that *C3-split* variants are usually expressed in both left and right valves of the same individual supports the presence of heritable variation, at least for this specific variant. Moreover, the evolutionary fixation of two variants also implies that the variants could be heritable. At the same time, several lines of evidence support environmental modulation on the expression of fossae variation. Okada (1982b) noted that fossae variants were elevated in laboratory populations of *Bicornucythere bisanensis* exposed to adverse conditions relative to populations in nature. Similarly, fossil populations of *B. bisanensis* were found to be much less variable in fossae patterns than extant populations (Abe 1983), a difference that might relate to pollution in the modern environment. The weak correlation in *Poseidonamicus* between depth and variation is also consistent with environmental modulation of fossae variability. Food availability and temperature both decrease with depth in the deep sea, and both of these factors might prove physiologically challenging to ostracodes.

Thus, there is evidence for both heritable and nonheritable sources of variation in fossae arrangement. This is consistent with Liebau's (1975) suggestion that left-right fossae variation within individuals parallels variation among individuals. Absent breeding studies, however, it is difficult to know more precisely the breakdown of genetic and environmental causes for fossae variation.

Regardless of the genetic basis of fossae variation, fitness estimates of the *C3-split* variant suggest that stabilizing or normalizing natural selection may also contribute to evolutionary conservation. The biological reasons for such fitness differences remain obscure. The resulting minor differences in ridge structure could conceivably influence the mechanical properties of the shell, but it is perhaps more likely that these cell divisions are correlated with other events necessary for normal development (i.e., internal selection sensu Wagner and Schwenk 2000). It should be noted, however, that evidence for stabilizing selection is limited to the *C3-split* variant. Even for this variant the evidence for fitness differences is not overwhelming, and some fraction of fossae variation may well be selectively neutral. This scenario is potentially consistent with the persistence of abundant variation in this character suite, which otherwise should be reduced by normalizing selection.

Structured variation and evolution

Surface ornament in macroreticulate ostracodes is expressed over a template of underlying epidermal cells, and the development of these cells constrains the morphology of the surface ridges. In *Poseidonamicus*, the resulting phenotypic variation turns out to be highly nonrandom: certain variants, and kinds of variants, are much more frequent than others. For example, *C3-split* is by far the most abundant fossae variant in this genus, whereas other kinds of variations, especially in fossae near the adductor muscle scars, are extremely rare. These variants may be seldom observed because the relevant cell divisions are more robust to genetic and environmental perturbation, or because whatever variations do occur incur higher fitness costs such that the ostracodes usually die before molting and shell mineralization are completed. Either way, the result is a highly skewed distribution of variation upon which selection and drift may act.

At least for the fossae examined here, much of the difference in variation among fossae was accounted for by the ontogenetic stage in which the fossae form: fossae that finalized in earlier instars were more consistent than those finalized in later instars. Increased variation through ontogeny is known from other biological systems. For example, Wood et al. (2007) reported that late-erupting teeth showed greater morphometric variation than early-erupting teeth in the condylarth *Ectocion osbornianus*. In a survey of morphological, life history and fitness-related traits in *Drosophila melanogaster*, Houle (1998) demonstrated similar elevated levels of mutational variability (phenotypic variation added by mutation each generation) in traits expressed in later ontogenetic stages. Houle suggested this pattern could be caused by later ontogenetic events being causally downstream from earlier events, and therefore influenced by a greater number of mutations than those occurring earlier in ontogeny. This mechanism can be related to ideas such as developmental burden (Riedl 1978; Wagner and Laubichler 2004) and generative entrenchment (Wimsatt and Schank 2004).

Although they are few in *Poseidonamicus*, evolutionary transitions in fossae arrangement do replicate the two most common intraspecific variations (*C3-split*, *C6/C7-split*). Such an association between variation within populations and divergence among populations has been observed in traits that are continuous (Mitchell-Olds 1996; e.g., Schluter 1996; Ackermann and Cheverud 2002; Blows and Higgie 2003; Hansen et al. 2003; Bégin and Roff 2004; Marroig and Cheverud 2005; Renaud et al. 2006; Hunt 2007a) and discrete (Shubin et al. 1995; Arthur and Farrow 1999). These studies, along with the data presented here, emphasize the potentially important evolutionary role that developmentally structured phenotypic variation can play (Maynard Smith et al. 1985; Arthur 2001, 2002; Brakefield 2006; Hendrikse et al. 2007). A natural extension of the present work would entail broadening the phylogenetic scope so as to encompass a larger sample of evolutionary transitions in the sequence of epidermal cell divisions.

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