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A GENERAL FRAMEWORK FOR THE ANALYSIS OF PHENOTYPIC TRAJECTORIES IN EVOLUTIONARY STUDIES

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Many evolutionary studies require an understanding of phenotypic change. However, while analyses of phenotypic variation across pairs of evolutionary levels (populations or time steps) are well established, methods for testing hypotheses that compare evolutionary sequences across multiple levels are less developed. Here we describe a general analytical procedure for quantifying and comparing patterns of phenotypic evolution. The phenotypic evolution of a lineage is defined as a trajectory across a set of evolutionary levels in a multivariate phenotype space. Attributes of these trajectories (their size, direction, and shape), are quantified, and statistically compared across pairs of taxa, and a summary statistic is used to determine the extent to which patterns of phenotypic evolution are concordant across multiple taxa. This approach provides a direct quantitative description of how patterns of phenotypic evolution differ, as well as a statistical assessment of the degree of repeatability in the evolutionary responses to selection among taxa. We describe how this approach can quantify phenotypic trajectories from many ecological and evolutionary processes, whose data encode multivariate characterizations of the phenotype, including: phenotypic plasticity, ecological selection, ontogeny and growth, local adaptation, and biomechanics. We illustrate the approach by examining the phenotypic evolution of several fossil lineages of *Globorotalia.*

KEY WORDS: **Adaptive diversification, morphological evolution, phenotypic change, residual randomization, phenotypic plasticity, ontogeny.**

Understanding how ecological selection generates changes in phenotypic traits is a major goal in evolutionary biology. Frequently, evolutionary biologists examine microevolutionary patterns among populations that span an ecological gradient to determine how divergent selection pressures influence phenotypic variation among localities. Through this approach, myriad examples of repeated evolution across common selective regimes have been identified, providing strong evidence of the adaptive process. For example, fishes in postglacial lakes frequently evolve distinct body forms when found in benthic and limnetic environments (Schluter and McPhail 1992; Robinson et al. 1993; Schluter 2000; Jastrebski and Robinson 2004), and reptiles that utilize distinct niches evolve consistent morphological differences on different islands ("ecomorphs": Losos 1992; Losos et al. 1998; Harmon and Gibson 2006). Other vertebrate examples are found in prey species that evolve distinct body types and life history traits in the presence of their primary predators (Reznick et al. 1996; Langerhans and DeWitt 2004; Langerhans et al. 2004, 2007), and species that evolve morphological differences in sympatry (character displacement) as a result of their competitive interactions (Schluter and

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McPhail 1992; Adams and Rohlf 2000; Adams 2004). These, and other, examples demonstrate an association between morphology and habitat, and provide a quantitative link between ecological selection and phenotypic evolution across environments.

Changes in phenotypic traits are the result of both external factors (e.g., changes in environmental condition) and internal factors (e.g., changes in genetic correlations or the genetic architecture underlying phenotypic traits). The microevolutionary consequences of both external and internal factors are often described through quantitative genetics approaches (Falconer 1960; Lande 1979; Lande and Arnold 1983). Within-taxon covariance matrices can be examined to determine whether angular differences of multivariate phenotypic evolution across populations or generations are observed (e.g., Arnold 1981; Phillips and Arnold 1999; Mezey and Houle 2003). Covariance matrices are calculated from phenotypic values from natural populations or additive genetic values in breeding experiments, and angles between the expected direction of phenotypic change, whether expressed by the dominant eigenvectors of the additive genetic covariance matrices (Phillips and Arnold 1999), or by vectors of differences between phenotypic means, can be statistically evaluated to test hypotheses of parallel evolution (e.g., Schluter 1996; Baer and Lynch 2003; Bégin and Roff 2003; Marroig and Cheverud 2005; see also Hansen and Houle 2008). Similarly, vectors describing phenotypic differences across populations can be used to compare the amount and direction of phenotypic evolution (Schluter 1996; Marroig and Cheverud 2005), and directions of phenotypic covariation across multiple developmental stages or time steps are compared in an analogous fashion (e.g., Badyaev and Martin 2000; Klingenberg et al. 2001). Phenotypic differences can also be described using the multivariate phenotypic means, from which the amount of phenotypic evolution between groups can be estimated (e.g., Hendry and Kinnison 1999; Adams and Rohlf 2000; Adams 2004). Another common approach is to examine patterns of contemporary microevolution across two distinct levels, such as benthic versus limnetic, predator versus nonpredator, allopatry versus sympatry, or from ancestor to descendant populations in an evolutionary or fossil sequence. Here, phenotypic differences across levels are commonly described using summary axes such as canonical or principal component scores (Jastrebski and Robinson 2004; Langerhans and DeWitt 2004).

The approaches mentioned here allow an assessment of patterns of phenotypic evolution across two evolutionary levels. In other systems, however, phenotypic evolution may occur across multiple evolutionary levels that form a sequence. Such sequences represent a phenotypic trajectory of evolutionary change. Recent theoretical work linking microevolutionary dynamics to macroevolutionary trends explore how phenotypic trajectories are generated from stepwise processes (Jones et al. 2007; Hohenlohe and Arnold 2008; Polly 2008). These studies provide considerable insight into how phenotypic trajectories are generated across multiple evolutionary time periods. Despite these advances, however, approaches for quantifying evolutionary sequences across multiple levels are less developed.

Recently, we developed an analytical procedure that quantifies patterns of phenotypic evolution between two evolutionary units (e.g., populations in different environments) across pairs of lineages or taxa (Adams and Collyer 2007; Collyer and Adams 2007). We describe phenotypic evolution as a phenotypic change vector (PCV). The magnitude of phenotypic change and its direction in phenotype space are then quantified, and these attributes are compared across pairs of taxa for an assessment of the extent to which phenotypic evolution is concordant across taxa (for quantitative genetics studies, PCV's are equivalent to $\mathbf{\Delta}\overline{\mathbf{Z}}$ in the breeder's equation: $\mathbf{\Delta}\overline{\mathbf{Z}} = \mathbf{G}\boldsymbol{\beta}$). In the present paper, we provide two important extensions of this approach that greatly expand its utility for evolutionary studies. First, we generalize the procedure to measure phenotypic evolution across multiple levels (i.e., an evolutionary sequence), allowing evolutionary patterns to be characterized as phenotypic trajectories, rather than phenotypic vectors. Second, we provide a summary statistic to assess the relative commonality of the evolutionary responses to selection for multiple lineages, thus providing a specific assessment of the extent to which the evolutionary process is repeatable. The utilization of the procedure for additional biological hypotheses is also discussed.

Quantifying Phenotypic Change across Two Levels

With multivariate data, phenotypic evolution is expressed in a trait space that is defined by the set of measured phenotypic characteristics (e.g., morphology, life history, or behavior). For a given lineage or taxon (e.g., species), evolutionary divergence can be defined as the difference in phenotype expressed between two levels in an evolutionary sequence. These levels could represent ancestor-descendent phenotypes, as in the case of fossil and extant taxa, or the difference between an extant taxon and its estimated ancestor along a phylogenetic tree (e.g., Revell et al. 2007). Alternatively, they could represent contemporary patterns of phenotypic evolution, expressed as the phenotypic differences between populations in differing environments (e.g, benthic vs. limnetic: Schluter and McPhail 1992, predator versus nonpredator: Reznick et al. 1996; Langerhans and DeWitt 2004). In all cases, the observed phenotypic evolution is a vector (Fig. 1A) connecting the phenotypic means from the two evolutionary levels (Schluter 1996; Baer and Lynch 2003; Bégin and Roff 2003; Collyer and Adams 2007). Mathematically, these vectors can be fully described by two attributes; the magnitude of phenotypic

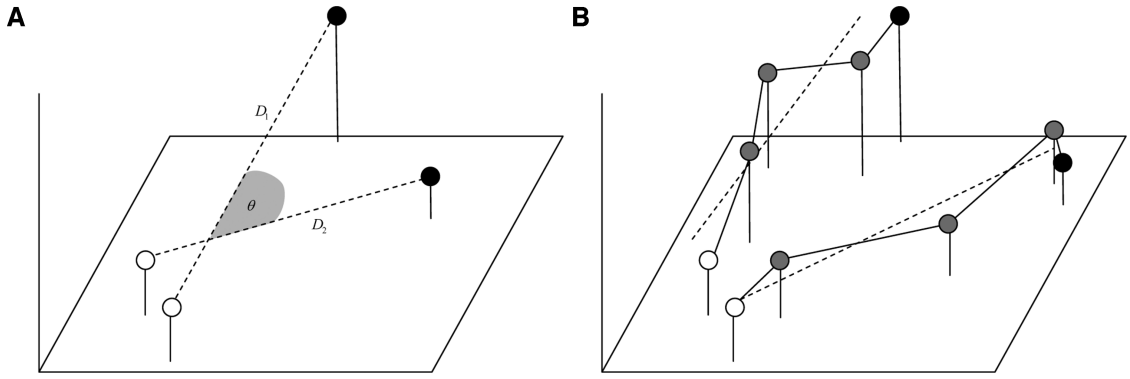


Figure 1. **(A) Phenotypic evolution vectors and their attributes of magnitude (*D*) and direction (θ) in phenotype space (B) Phenotypic evolution trajectories for two species across multiple evolutionary levels. Each evolutionary sequence begins with populations represented in white, and terminates with populations represented in black. The summed path-length across sequential levels represents the magnitude of phenotypic evolution, the overall direction of phenotypic evolution is represented as the dashed line, and the shape of the phenotypic trajectory can also be visualized.**

change, and the direction of phenotypic change in the phenotypic data space. Understanding similarities and differences in patterns of phenotypic evolution is equivalent to determining the extent to which these attributes are concordant. Patterns of phenotypic change are examined by quantifying the attributes of PCV vectors (magnitude and direction), and statistically comparing these attributes among multiple species (Collyer and Adams 2007).

To assess concordance in phenotypic change attributes, observed patterns are compared to empirically generated random distributions. These are frequently obtained from bootstrap methods, where confidence intervals are used to determine whether vector attributes among species are concordant (e.g., Schluter 1996; Bégin and Roff 2003), or from permutation procedures, where experimental units are randomized across treatments (e.g., Klingenberg et al. 2004). Another useful approach is to randomize residuals from reduced linear models to assess significance levels (Freedman and Lane 1983; Gonzalez and Manly 1998; Collyer and Adams 2007). Such "residual randomization" methods obtain estimated phenotypic values from two models: a model containing effects for species (or other taxonomic variables), evolutionary levels (e.g., ancestor or descendent), and the interaction of these effects; and a reduced model containing only effects for species and evolutionary levels (i.e., lacks the interaction of these effects). Residuals from the reduced model are randomly shuffled onto estimated values to obtain pseudo-values (see Appendix). In each iteration of the randomization procedure, pairwise differences in magnitude and direction of PCVs are calculated (holding main effects constant), and the observed values are compared to distributions of random values to assess their significance (Collyer and Adams 2007). A key advantage of this approach is that it accounts for variation due to covariates and other non-targeted sources of variation with no procedural alteration (see discussion in Adams and Collyer 2007). The procedure is fully discussed in the Appendix.

Phenotypic Change across Multiple Levels: Trajectory Analysis

Despite its utility, a limitation of the phenotypic change vector method is that it is only useful for quantifying evolution between two evolutionary levels, while some systems (such as fossil sequences or populations along an ecological gradient), may have multiple levels. However, the approach can be easily generalized for quantifying phenotypic change across multiple evolutionary time steps or other evolutionary levels. Procedurally, this generalization treats phenotypic change as a multivariate trajectory that links a sequence of points (evolutionary levels) in phenotype space (Fig. 1B). Like phenotypic change vectors, these evolutionary trajectories have a size (magnitude) and an orientation (direction) in phenotype space. However, they also have the additional attribute of shape. Thus, patterns of phenotypic change are examined by determining the phenotypic evolution trajectories for each group (e.g., species), quantifying their attributes (magnitude, direction, and shape), and statistically evaluating the differences in attributes to infer how patterns of evolutionary divergence differ among taxa.

Phenotypic evolution trajectories can be defined as the ordered-sequence of estimated phenotypes along the evolutionary path for each species. Trajectory size is then defined as the path-length distance along the evolutionary trajectory, found from the set of Euclidean distances between sequential evolutionary levels. Trajectory orientation is described as the direction of first principal component (PC1) of the covariance matrix estimated

from the trajectory points, standardized by the starting (ancestral) point. Finally, differences in trajectory shape are found from the deviations between corresponding evolutionary levels across two scaled and aligned phenotypic trajectories, expressed as Euclidean distance (D_{Shape}). The analytic procedure for obtaining differences in trajectory shape uses a least-squares superimposition alignment analogous to that used for landmark configurations in geometric morphometric methods. The approach is described in the Appendix. One appealing aspect of the approach described above is that PCVs are simply two-point trajectories that can be analyzed the same way. That is, the definition of trajectory size is the same, the linear equation that describes PC1 for a two-point trajectory is mathematically identical to the PCV itself, and there is no difference in shape between two vectors, so the attribute of shape simply does not apply. Therefore, the computational steps used to calculate trajectory attributes inherently produce PCV attributes when only two evolutionary levels are examined.

Evolutionary Interpretations of Trajectory Attributes

It is now worth considering what the attributes of phenotypic trajectories represent in evolutionary terms. Trajectory size quantifies the path length of the evolutionary trajectory expressed by a particular taxon across evolutionary levels. This represents the actual amount, or magnitude, of phenotypic evolution displayed by that taxon. Thus, if two evolutionary trajectories differ only in their path length (i.e., their orientation and shape are identical), the differences in phenotypic evolution can be described as one species exhibiting greater phenotypic evolution relative to that of another. If divergence times are known, trajectory size can be converted to standard measures of evolutionary change (Haldane 1949; Gingerich 1993). For instance, one can calculate darwins by dividing trajectory size by time (sensu McPeek et al. 2008; see also Felsenstein 1988), or calculate haldanes by estimating generalized trajectory size from trajectories standardized by the pooled within-group covariance (sensu Haldane 1949; Gingerich 1993). Thus, if the trajectories of two or more taxa are compared over comparable time periods (e.g., two lineages with equal generation times from a common ancestor), differences in trajectory size indicate differences in rates of evolution. Because rates of evolution tend to decline proportional to time however (Gingerich 1993), differences in trajectory size only correspond to differences in rates of evolution for studies where time is known or can be estimated.

The second attribute to consider is trajectory direction. Trajectory direction describes the general orientation of phenotypic evolution in the multivariate trait space. In some circumstances, statistical comparisons of trajectory direction can be used to provide an assessment of patterns of convergence, divergence, and parallelism. For example, when two trajectories are oriented in a similar direction, parallel evolution (sensu Schluter 1996; Stayton 2006) is identified. On the other hand, when a hypothesis of parallel evolution is rejected, either convergence or divergence may be identified.

The third attribute of phenotypic evolution trajectories is trajectory shape. This attribute describes the shape of the path of phenotypic evolution through the multivariate trait space. Phenotypic trajectories may show simple evolutionary paths (such as a linear or slightly curve-linear path across evolutionary levels), or may be more complex (e.g., showing oscillations in phenotypes). Trajectory shape is an attribute that can identify the recurrent expression of different phenotypes in a taxon's evolutionary history, rather than its gradual change from one form to another. The shape of the path of phenotypic evolution represents a potentially informative attribute of phenotypic evolution that similarly requires investigation. Its value in paleontological stratigraphic sequences can be appreciated because points in the trajectories of different taxa may correspond directly to known geological time periods. Further, its value in microevolutionary studies may be found through multi-generational studies, where the points in trajectories correspond to generation intervals.

The attributes of evolutionary trajectories represent quantitative measures for which statistical tests can be independently applied. Nonetheless, it is the combination of similarities and differences across trajectory attributes that define the nature of phenotypic evolution differences between taxa. An empirical example of this was recently described in *Plethodon* salamanders in the southern Appalachian Mountains, where competition drives phenotypic divergence (Adams 2004). It was discovered that the phenotypic evolutionary response of two species to competition was similar in magnitude, but the sympatric populations evolved in different directions in phenotype space, consistent with a hypothesis of character displacement (Collyer and Adams 2007). Therefore, the observed sympatric differences in this system were the result of both species evolving different cranial features, but the overall amount of phenotypic evolution was consistent between the two taxa (i.e., both species responded equally to competition). In another example (presented below), the evolution of two different species of the planktonic *Globorotalia* were found to have similar overall directions of phenotypic evolution, but the trajectory shapes, and consequently, the amount of phenotypic change, differed over five geologic time periods, characterized by greater evolutionary stasis exhibited by one species in recent time. These examples highlight that examining different attributes of phenotypic trajectories provides a richer description of patterns of phenotypic evolution, and thus considerable insight into our understanding of evolutionary patterns.

Summarizing Phenotypic Change across Multiple Evolutionary Trajectories

The approach above directly quantifies attributes of patterns of phenotypic evolution across two or more evolutionary levels. However, statistical assessments comparing attributes of phenotypic evolution are accomplished in pairwise fashion. Therefore, when more than two lineages or taxa are examined, a series of comparisons must be performed (e.g., Hollander et al. 2006; Chun et al. 2007). Although this "pairwise comparison" approach is useful for specific species-species comparisons, it does not provide an overall assessment of the relative similarity of phenotypic change across the entire set of evolutionary trajectories. Thus, quantitatively assessing the concordance of evolutionary change remains a challenge.

To provide an overall test, we propose the following procedure. First, the size, direction, and shape of each phenotypic evolution trajectory are determined for a set of m taxa. Next, all pairwise comparisons of differences in trajectory attributes are calculated. For example, the differences in magnitude, orientation, and shape between the first and second trajectories in a data set are found as: $MD_{1,2}$, $\theta_{1,2}$, and $D_{\text{Shape:1,2}}$ (see Appendix for derivations). For convenience, these can be assembled across all pairwise comparisons into matrices expressing the differences in magnitude, direction, and shape. A summary statistic is then calculated for each trajectory attribute that expresses the variation in differences across the set of trajectories. For example, the summary statistic for differences in trajectory size is found as:

$$\text{Var}_{\text{Size}} = \frac{\sum_{i}^{m(m-1)/2} (MD_i - \overline{MD})^2}{(m(m-1)/2) - 1} \quad (1)$$

where m is the number of taxa (trajectories), MD_i is the difference in magnitude for the ith comparison of phenotypic trajectories, and $\overline{MD}$ is the mean difference in trajectory magnitude. Similar statistics are obtained for differences in trajectory orientation and differences in trajectory shape.

The summary values provide a quantitative estimate of the relative similarity of evolutionary responses across a set of trajectories, expressed in trajectory magnitude, trajectory orientation, and trajectory shape. These values are used as test statistics and can be evaluated using, for example, the same permutation procedure used to evaluate pairwise differences. If a set of trajectories are dissimilar in one or more of these attributes, the observed summary statistic will be greater than expected from random chance. This simple procedure provides a quantitative estimate of the relative similarity of evolutionary responses across a set of trajectories, and thus provides a specific assessment of the extent to which evolutionary patterns are repeatable.

The test statistics proposed here are appropriate for determining the extent to which evolutionary trajectories differ in their individual attributes (size, direction, and shape). However, other null expectations from evolutionary models exist that are also of interest to examine quantitatively. For example, patterns of phenotypic change in paleontological sequences are frequently compared to what is expected under a random walk or to patterns expected under directional evolutionary change (e.g., Bookstein 1987; Hunt 2006). The analysis of trajectory size, shape, and direction provides a complementary approach to examining phenotypic evolution through time, and could be combined with neutral evolutionary models for a more comprehensive assessment of patterns of phenotypic evolution.

Examples

Here we provide two simulated examples and one empirical example that demonstrate the utility of the trajectory analysis approach (computer code for performing the analyses, along with simulated data sets, are found in the online Supporting Information). The first simulated example represents phenotypic data from three species at two evolutionary levels. The data are shown in Figure 2A. This data represents the contemporary evolution that occurs when a species from one environment invades a novel environment; though they could also represent time points such as ancestor-descendant populations (see above). We simulated the data such that the phenotypic evolution across levels for two species (A and B) was in the same direction, but differed in magnitude, while the phenotypic evolution across levels for the third species (C) was of the same magnitude as the first species, but differed in its direction in phenotype space. For each species × environment mean, 30 random values (specimens) were generated using a model of isotropic error (for additional empirical examples see Collyer and Adams 2007). The data were analyzed using the residual randomization method (Freedman and Lane 1983; Gonzalez and Manly 1998; Collyer and Adams 2007), with residuals from a model that lacked species × environment effects (i.e., species and environment effects were held constant). All analytical steps were performed in R (R Core Development Team 2008).

For this example, a multivariate analysis of variance (MANOVA) revealed significant variation among species (Pillai's trace = 1.02, $F_{4,348} = 89.98$, $P < 0.0001$), between environments (Pillai's trace = 0.95, $F_{2,173} = 1{,}677.80$, $P < 0.0001$), as well as in the species × environment interaction (Pillai's trace = 1.34, $F_{4,348} = 176.91$, $P < 0.0001$). Using the trajectory approach (with 10,000 residual randomization permutations) we correctly found that the direction of phenotypic evolution across environments was not different for species A and B ($\theta_{A,B} = 1.794^\circ$, $P = 0.7579$), but that these two species did differ significantly in the

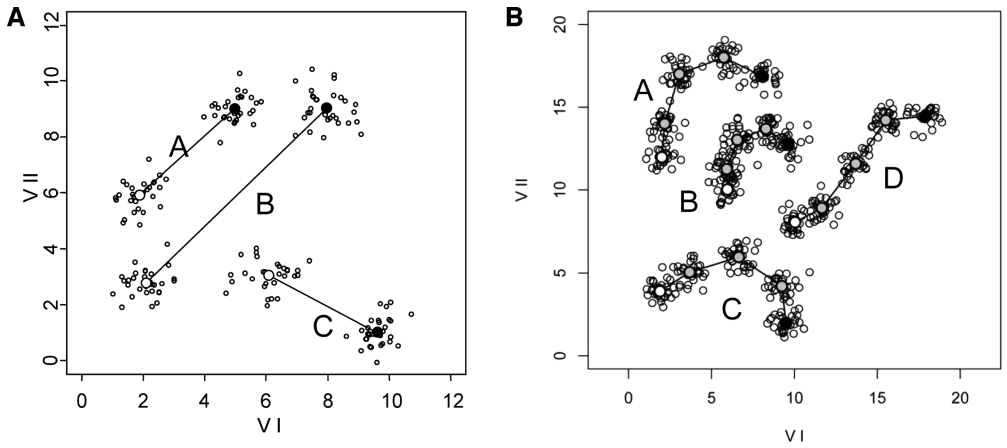


Figure 2. Simulated phenotypic evolution for (A) three species across two evolutionary levels, and (B) four species across five evolutionary levels. Evolutionary levels could correspond to ancestral-descendent time points, points in a fossil sequence, or populations across an environmental gradient (representing contemporary evolution). In both panels, open circles represent randomly generated individuals (30 per species × environmental level) generated from normal multivariate error at each species × evolutionary level combination. For phenotypic vectors (A), vectors A and B are orientated in the same direction but are of different magnitudes, and vector C is of the same magnitude as vector A, but is oriented differently. For phenotypic trajectories (B), trajectory B differs from the others in size, trajectory C differs from the others in orientation, and trajectory D differs from the others in shape. Starting points are shown in white, intermediate points in gray, and end points in black.

amount of phenotypic evolution exhibited ($MD_{A,B} = 4.2105$, $P = 0.0001$). By contrast, the direction of phenotypic evolution across environments for species C was significantly different from that of the species A ($\theta_{A,C} = 74.69777^{\circ}$, $P = 0.0001$), but the magnitude of phenotypic evolution did not differ between them ($MD_{A,C} = 0.2854$, $P = 0.5752$). The summary statistics revealed that there was significant variation in the direction of evolutionary change across the three taxa ($\text{Var}_{\text{orient}} = 1816.32$, $P = 0.0001$), as well as significant variation in the amount (magnitude) of phenotypic evolution ($\text{Var}_{\text{size}} = 5.536$, $P = 0.0001$). Therefore, in this example, little overall concordance was revealed in the phenotypic evolution exhibited by the three taxa. Finally, because we simulated these data with known patterns of phenotypic evolution, we confirmed that the trajectory approach described above was capable of identifying differences in patterns of phenotypic evolution across taxa when they were known to be present, and did not identify differences when they were not present.

The second simulated example represents a more complicated scenario, where phenotypic evolution is observed for four species across five evolutionary levels, such as may be found in a fossil series or other evolutionary sequence. The data for this example are shown in Figure 2B. We simulated these data such that the magnitude of phenotypic evolution was different for one species (B) relative to the others, the direction of phenotypic evolution was different for one species (C) relative to the others, and the shape of the phenotypic evolutionary trajectory was different for one species (D) relative to the others. As with the previous example, for each species × evolutionary level mean, 30 random values (specimens) were generated using a model of isotropic error. Data were analyzed as described above.

With this example, a MANOVA revealed significant variation among species (Pillai's trace = 2, $F_{6,1160} = 10{,}860.6$, $P < 0.0001$), between evolutionary levels (Pillai's trace = 1.7, $F_{8,1160} = 711.8$, $P < 0.0001$), and in their interaction (Pillai's trace = 1.5, $F_{24,1160} = 164.6$, $P < 0.0001$). Using the trajectory approach (with 10,000 residual randomization permutations), we found significant differences in trajectory magnitude, trajectory direction, or trajectory shape when they were known to be present, and did not find differences when trajectories were known to be similar (Table 1). Additionally, summary statistics revealed that there was significant variation in the direction of evolutionary change across the four taxa ($\text{Var}_{\text{orient}} = 884.312$, $P = 0.0001$), significant variation in the amount (magnitude) of phenotypic evolution ($\text{Var}_{\text{size}} = 4.21$, $P = 0.0001$), and significant variation in the shapes of the phenotypic trajectories ($\text{Var}_{\text{shape}} = 0.0199$, $P = 0.0001$). Therefore, these findings revealed a general lack of concordance across the set of trajectories in their direction, magnitude, and shape, which was due in large part to the fact that one phenotypic trajectory differed greatly from the remaining trajectories in each trajectory attribute.

As a final example, we examined patterns of phenotypic evolution in two fossil species of *Globorotalia*. The data were part of a larger study in which phenotypic and phyletic patterns of evolution through time were examined for several species in multiple ocean basins (Spencer-Cervato and Thierstein 1997). For this example, a total of 907 specimens from two species, *G. crassaformis*

Table 1. Statistical assessment of differences in phenotypic trajectory size ($MD_{1,2}$), direction ($\theta_{1,2}$), and shape (D_{Shape}) differences of four phenotypic evolutionary trajectories (A, B, C, and D) from Figure 2B. Bolded values are those expected to be different based on the simulation of data. Observed significance levels (*P*-values) were empirically generated from 10,000 random permutations as described.

Comparison	$MD_{1,2}$	P_{Size}	$\theta_{1,2}$	P_{θ}	D_{Shape}	P_{Shape}
A,B	**3.7866**	**0.0001**	0.0367	0.9927	0.0878	0.7491
A,C	0.0700	0.8953	**54.6515**	**0.0001**	0.0332	0.9982
A,D	0.2018	0.7120	1.1431	0.7649	**0.3011**	**0.0001**
B,C	**3.8566**	**0.0001**	**54.6883**	**0.0001**	0.0855	0.7784
B,D	**3.9884**	**0.0001**	1.1063	0.7735	**0.3576**	**0.0001**
C,D	0.1318	0.8131	**55.7946**	**0.0001**	**0.3065**	**0.0001**

($N = 369$) and *G. tosaensis* ($N = 538$), were obtained from a single deep-sea core located in the south Pacific (see Spencer-Cervato and Thierstein 1997 for details). Fossil specimens from each species were identified from five distinct time layers from the Pliocene and Pleistocene (3.28, 3.06, 2.79, 2.60, and 2.31 million years ago). For each specimen, two measurements were chosen (top view perimeter; and side view box area ratio: described in Lazarus et al. 1995; Spencer-Cervato and Thierstein 1997). Our analyses were therefore based on a two-factor model with species and time layers as main effects, along with their interaction.

Using MANOVA, we found significant morphological differences among species (Pillai's trace = 0.171, $F_{2,896} = 92.553$, $P < 0.0001$), between time layers (Pillai's trace = 0.135, $F_{8,1794,} = 16.242$, $P < 0.0001$), as well as in the species × time layer interaction (Pillai's trace = 0.109, $F_{8,1794} = 12.879$, $P < 0.0001$). Using the trajectory approach (with 10,000 residual randomization permutations), we found significant differences in the magnitude of phenotypic evolution between the two species ($MD_{1,2} = 73.30$, $P_{size} = 0.0364$), implying that one species exhibited a greater rate of phenotypic evolution relative to the other. In addition, when the path lengths of each phenotypic evolution trajectory were standardized by time, we found that *G. tosaensis* displayed considerably more phenotypic evolution per unit time than did *G. crassaformis* (i.e., *G. tosaensis* exhibited 21% greater morphological evolution per million years than *G. crassaformis*). We also found differences in the shape of the evolutionary trajectories through time ($D_{Shape} = 0.544$, $P_D = 0.006$). However, there were no differences in the overall direction of the two evolutionary trajectories ($\theta_{1,2} = 0.0033$, $P_{\theta} = 0.6370$). When viewed as phenotypic trajectories through evolutionary time (Fig. 3), these statistical conclusions were graphically confirmed. Specifically, the phenotypic evolution trajectory of *G. tosaensis* displayed

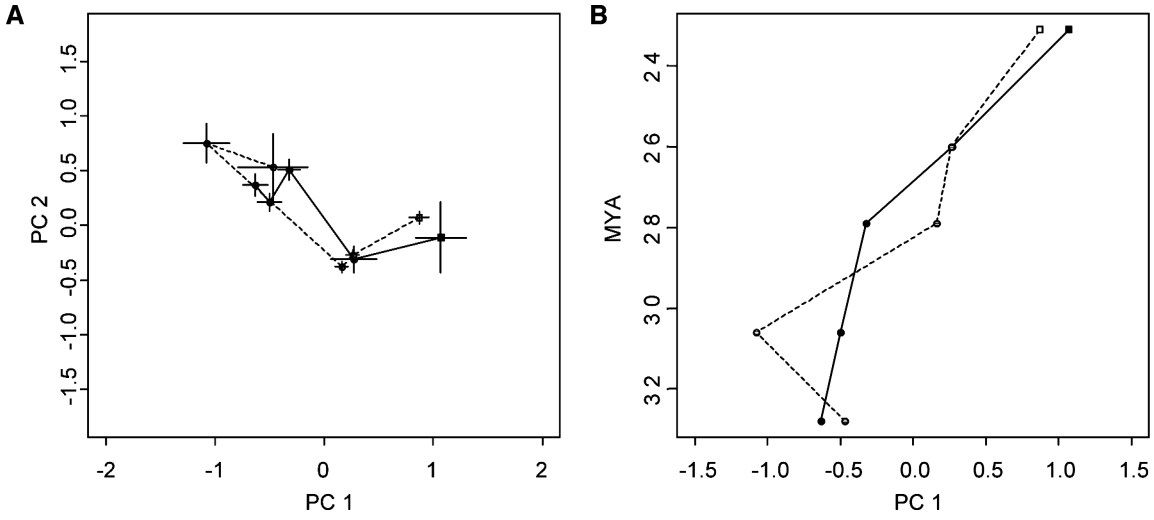


Figure 3. (A) Plot of principal components estimated from the correlation matrix for *Globorotalia* phenotypic data. Because the data were originally bivariate, this plot shows 100% of the phenotypic variation for standardized data (PC 1 accounts for 62% of the overall variation). Error bars represent one standard error of species × time period means. Phenotypic trajectories for *G. crassaformis* (solid) and *G. tosaensis* (dashed) through the Pliocene are shown as connecting lines. (B) Alternative representation of phenotypic evolution trajectories scaled by time. Phenotypic values are represented by the means on first PC axis in (A). Fossils were sampled at five time periods from the Pliocene (3.28, 3.06, 2.79, 2.60, and 2.31 million years ago). *G. crassaformis* values are black and *G. tosaensis* are white in each plot. Squares indicate most recent values (2.31 million years ago).

considerably greater evolutionary change from time period to time period as compared to the phenotypic evolution trajectory of *G. crassaformis*. Additionally, this plot revealed that the evolutionary changes through time were not gradual and in a single direction, but rather fluctuated through time, as may be expected from a lineage undergoing random evolution or stabilizing selection (Polly 2004). This was especially the case for *G. tosaensis,* whose phenotype oscillated greatly along the major morphological axis (PC1). Finally, while there was considerable phenotypic evolution exhibited by both species, their phenotypes at both the earliest (3.28 million years ago) and latest (2.31 million years ago) time periods were quite similar to one another.

Discussion

A major goal in evolutionary biology is to understand patterns of phenotypic evolution. However, while many approaches exist for examining patterns of variation across pairs of evolutionary units, methods for assessing phenotypic changes across a sequence of multiple evolutionary levels are less developed. In this paper, we described a general analytical procedure for quantifying and comparing multivariate patterns of phenotypic evolution. The approach builds on prior evolutionary work (e.g., Schluter 1996; Phillips and Arnold 1999; Bégin and Roff 2003; Marroig and Cheverud 2005; Adams and Collyer 2007; Collyer and Adams 2007), where the amount and direction of contemporary evolution is quantified in various ways, and compared among taxa or groups. With our approach, patterns of phenotypic evolution are quantified as a trajectory through the phenotypic trait space, the attributes of phenotypic evolution trajectories (their size, direction, and shape) are then quantified, and statistically evaluated to identify similarities and differences in patterns of phenotypic evolution across taxa. Through simulated examples, we demonstrated that comparisons of phenotypic evolution trajectories were capable of identifying differences in patterns of phenotypic evolution across taxa when they were known to be present, and did not identify differences when they were known to be absent. The phenotypic evolution among two fossil lineages of *Globorotalia* served as an empirical example of the approach.

A key benefit of evaluating phenotypic evolution trajectories is that this approach quantifies phenotypic divergence directly, rather than using indirect inference from variance components and plots from summary axes such as canonical axes. That is, phenotypic trajectories quantify the actual path of phenotypic evolution for each taxon (described by the difference in phenotypic means across evolutionary levels) and therefore, are a quantitative description of how patterns of phenotypic evolution are expressed. Further, the attributes describing these trajectories have direct evolutionary interpretation, and can be used to evaluate the types of evolutionary responses we wish to identify (e.g., differences in the rate of phenotypic evolution, evolutionary convergence, etc.). As such, statistical comparisons of these attributes provide a more rigorous quantification of how phenotypic patterns are similar or different among taxa, and how the phenotype changes through evolutionary time.

The phenotypic evolution trajectory approach quantifies phenotypic change from a sequence of evolutionary levels, and dissects these patterns into their fundamental components (trajectory size, shape, and direction). Because each component is analyzed separately, the method can identify patterns where some attributes are similar for a set of evolutionary trajectories while others are different. In such cases, these similarities and differences provide precise biological description of the nature of phenotypic evolution for these taxa. They also reveal that patterns of phenotypic evolution are likely more complex than we have come to believe. For example, one long-standing question in evolutionary biology is determining whether common selective pressures generate similar phenotypic responses. However, if two species are found to exhibit phenotypic evolution in a similar direction but with different magnitudes, should one classify this as a common evolutionary response or a different evolutionary response? By contrast, if two species exhibit phenotypic evolution in different directions but with similar magnitude, how is this to be classified? Using phenotypic evolution trajectories, it is possible to identify such patterns, and in fact, the fossil example above revealed exactly that (differences in trajectory size and shape, but not direction). As a consequence, the analysis of phenotypic evolution trajectories can reveal important aspects of the diversification process that otherwise go undetected. This highlights the need to address such common questions as: "Is the evolutionary process repeatable?" using an analytical framework that can quantify such subtleties. We feel that addressing such questions with phenotypic evolution trajectories will reveal more complicated evolutionary patterns than have previously been detected, which may provide considerable insight into our understanding of the evolutionary process.

The analysis of phenotypic trajectories described here was motivated in terms of understanding patterns of phenotypic evolution across evolutionary levels. In the empirical example, we examined patterns of phenotypic evolution across distinct "time layers" in evolutionary sequences of fossils. In cases of contemporary microevolution, the approach could be used to assess patterns across levels representing populations in different environments along some ecological gradient (e.g., high salinity, medium salinity, low salinity, etc.). Despite the clear evolutionary perspective of these examples however, it is important to realize that the approach has much broader utility for describing other ecological and evolutionary patterns. In fact, the approach should be useful for describing patterns of change from any ecological

or evolutionary process that forms an ordered sequence of points in a multivariate data space. For example, the approach may be useful in studies of phenotypic plasticity to describe how plastic responses across environments differ among taxa (i.e., to describe patterns associated with a significant G × E interaction term). It may also be used for determining how allometric or ontogenetic growth trajectories differ, or for quantifying patterns in other data that form a time-sequence (but see approaches in Griswold et al. 2008). Patterns of biomechanical motion may be described using phenotypic trajectories, where the posture of individuals across multiple time steps during a motion represent an ordered sequence that defines a "motion trajectory" (see Adams and Cerney 2007). Finally, the approach is not restricted to the analysis of morphological data, but may also be used to evaluate multivariate patterns in other data, such as life history traits, gene expression, or behavioral traits, that vary across time steps or any other set of sequential units. When viewed in this light, the trajectory approach described here provides a general conceptual framework for understanding patterns of change in phenotypic or other quantitative data.

Understanding the evolution of phenotypic diversity has long been a major goal of much of evolutionary biology. However, quantitatively assessing the path of phenotypic evolution has been challenging, due in part, to lack of a common conceptual framework and tools for assessing and comparing such patterns. Recent advances provide analytical tools for assessing such changes across pairs of levels, such as the evolutionary path formed by ancestors and descendents on a phylogenetic tree (e.g., Revell et al. 2007). The phenotypic evolution trajectory approach described here provides a similar analytic solution for assessing evolutionary change across multiple evolutionary levels. We believe that the analysis of phenotypic evolution trajectories offers a useful analytic tool for examining patterns of evolutionary change across a sequence of levels, and thus enables us to better understand how patterns of phenotypic evolution are similar or different across taxa. Further, when combined with other approaches, one could quantitatively compare the observed path of evolutionary change to that predicted under sequential quantitative genetics models (e.g., Hohenlohe and Arnold 2008; Polly 2008). Thus, the ability to quantify patterns of phenotypic change to this fine degree provides an empirical base upon which additional levels of biological hypotheses emerge. As such, the goal of understanding the extent to which the evolutionary process is repeatable may be quantitatively realized.

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Appendix of Analytical Details

For the examples presented in this article, phenotypic means for each species × time step were obtained from linear models. Here we provide statistical details for the generalized Procrustes analysis used to obtain trajectory attributes, and demonstrate how these attributes can be statistically evaluated with residual randomization. Phenotypic values, $\mathbf{Y}$, can be expressed by a generalized linear model, $\mathbf{Y} = \mathbf{X}\boldsymbol{\beta} + \varepsilon$; where $\mathbf{X}$ is an $n \times k$ design matrix describing the k model effects for n objects, $\boldsymbol{\beta}$ is a $k \times p$ matrix of partial regression coefficients for p response variables, and $\boldsymbol{\varepsilon}$ is the $n \times p$ matrix of residuals. The matrix of residuals can also be written as $\boldsymbol{\varepsilon} = \boldsymbol{\Sigma}\boldsymbol{\sigma}^2$, where $\boldsymbol{\Sigma}$ is a covariance matrix that describes the nonindependence of error, if phylogenetic or spatial relatedness among observations is to be considered (see Adams and Collyer 2007). For a typical phenotypic change study, $\mathbf{X}$ takes the form of a two-factor MANOVA, one factor representing the m taxa or lineages (e.g., species), a second factor representing the l evolutionary levels (ancestor–descendent levels, fossils from a time sequence, etc.), and their interaction. We represent this as the design matrix for the full model, $\mathbf{X}_f$. Parameter estimates are obtained as the solution to the generalized least squares (GLS) problem: $\hat{\boldsymbol{\beta}}_{f(GLS)} = (\mathbf{X}_f^t \Sigma^{-1} \mathbf{X}_f)^{-1} \mathbf{X}_f^t \Sigma^{-1} \mathbf{Y}$, where ^{t} and $^{-1}$ correspond to matrix transpose and inverse, respectively. When residuals are independently distributed, this equation simplifies to: $\hat{\boldsymbol{\beta}}_f = (\mathbf{X}_f^t \mathbf{X}_f)^{-1} \mathbf{X}_f^t \mathbf{Y}$, because $\boldsymbol{\Sigma}$ is an identity matrix. Phenotypic values (LS means) that make up the phenotypic trajectory are estimated as: $\bar{\mathbf{Y}} = E[\mathbf{Y}|\mathbf{X}_f, \hat{\boldsymbol{\beta}}_f]$, where E is the expected value.

Phenotypic evolution trajectories are matrices partitioned from $\bar{\mathbf{Y}}$, such that $\bar{\mathbf{Y}} = \begin{bmatrix} \bar{\mathbf{Y}}_1 \\ \hline \bar{\mathbf{Y}}_2 \\ \hline \vdots \\ \hline \bar{\mathbf{Y}}_m \end{bmatrix}$ for m lineages, each with the number of rows equal to the number of points (l) in the trajectory (where l is the number of evolutionary levels). To obtain estimates of the size, orientation, and shape of evolutionary trajectories, the following procedures are performed.

(1) **Trajectory size**: Trajectory size is found as the path-length distance along the evolutionary trajectory. This is defined as the sum of the distances between adjacent evolutionary levels. For each trajectory, $\bar{\mathbf{Y}}_m$, its size is found as: $D_{size} = \sum_{j=1}^{l-1} \sqrt{(\bar{\mathbf{Y}}_{m,j} - \bar{\mathbf{Y}}_{m,(j+1)})^2}$. The test statistic describing pairwise differences in D_{size} is then calculated between taxa as: $MD_{1,2} = |D_{size1} - D_{size2}|$.

(2) **Trajectory orientation**: Trajectory orientation is described by the direction of its first principal component (PC1). For each trajectory, $\bar{\mathbf{Y}}_m$, PCA is performed. Pairwise angular differences are then obtained between first PCs of different trajectories. The vector correlation between taxa PCs is the inner product of the PCs, standardized by their Euclidean distances. The angle is the arccosine of this value: $\theta_{1,2} = \cos^{-1}(\frac{PC_{11}}{D_{PC_{11}}} \cdot \frac{PC_{12}^T}{D_{PC_{12}}})$. Finally, to ensure that $\theta_{1,2}$ properly incorporates the direction from ancestor to descendant, the starting point (ancestor) is projected onto its PC axis. The difference between the sign of these projected points is then used to determine whether $\theta_{1,2}$ or $(\pi - \theta_{1,2})$ should be used (see Adams and Cerney 2007).

(3) **Trajectory shape:** Trajectory shape corresponds to the relative configuration of points (evolutionary levels) expressed in the phenotypic data space. Describing the shape of a configuration of points is accomplished using Procrustes approaches. In evolutionary biology, Procrustes analysis is most commonly used for comparing anatomical shapes though geometric morphometrics (Bookstein 1991, Rohlf and Marcus 1993, Adams et al. 2004). However, Procrustes analysis is a general statistical standardization procedure that allows the comparison of shapes from any configurations of points, and is used in a wide variety of disciplines (for statistical description and examples see Gower and Dijksterhuis 2004). To obtain the shape of evolutionary trajectories, the following steps are performed: (a) Every trajectory, $\bar{\mathbf{Y}}_m$, is mean-centered by subtracting the overall species mean $(\bar{\mathbf{Y}}'_m = \bar{\mathbf{Y}}_m - \overline{\bar{\mathbf{Y}}}_m)$, such that all $\bar{\mathbf{Y}}'_m$ are centered in the same location (the origin of the data space). (b) Every $\bar{\mathbf{Y}}'_m$ is rescaled by dividing by its centroid size (CS), such that $\bar{\mathbf{Y}}_m = \frac{1}{CS}\bar{\mathbf{Y}}'_m$. Because each trajectory is already mean-centered, its centroid size is found as: $CS = (\bar{\mathbf{Y}}'_m \bar{\mathbf{Y}}''_m)^{1/2}$. (c) Every $\bar{\mathbf{Y}}''_m$ is optimally rotated in a least squares sense such that the variation among trajectories is minimized (see Rohlf and Slice 1990 for computational details). We refer to these matrices as $\bar{\mathbf{Y}}^*_m$, and the pairwise difference in shapes (Euclidean distance) between any two trajectories can be solved as $D_{Shape_{1,2}} = \left[\left(\bar{\mathbf{Y}}^*_1 - \bar{\mathbf{Y}}^*_2 \right) \left(\bar{\mathbf{Y}}^*_1 - \bar{\mathbf{Y}}^*_2 \right)^t \right]^{1/2}$.

To statistically examine trajectory attributes, one can evaluate the variation in the lineage × evolutionary level interaction in MANOVA. In MANOVA, the sums of square and cross-products (SSCP) matrix associated with the interaction is solved and a statistical test (e.g., Pillai's trace) is used to evaluate if the variance statistically differs from 0. This is accomplished by removing the parameters from the original model design matrix ($\mathbf{X}_f$) that describe the interaction to produce $\mathbf{X}_r$, and recalculating the matrix parameter estimates ($\hat{\boldsymbol{\beta}}_r$), where r refers to the reduced model. The SSCP is found as $\mathbf{SSCP} = \hat{\boldsymbol{\beta}}_f^t \mathbf{X}_f^t \mathbf{Y} - \hat{\boldsymbol{\beta}}_r^t \mathbf{X}_r^t \mathbf{Y}$. Collyer and Adams (2007) demonstrated that the statistical significance of the SSCP can be evaluated through residual randomization (Gonzalez and Manly 1998), where the rows of residuals from the reduced model ($\boldsymbol{\varepsilon}_r$) are randomized. For factorial designs, residual randomization also has superior statistical power as compared to alternative resampling approaches (see Anderson and ter Braak 2003). In each iteration of the residual randomization procedure, residuals are randomized and are added to predicted values to produce random values ($\mathbf{Y}^* = \hat{\mathbf{Y}}_r + \varepsilon_r^*$), such that nontargeted effects are held constant. $\hat{\boldsymbol{\beta}}_f$ is recalculated, which produces a random version of SSCP under the null hypothesis that the variance of the interaction is 0. By performing many iterations, an empirical null distribution of a test statistic for the SSCP is created, and the significance of the observed statistic is inferred from its percentile in the null distribution. This procedure can be considered a nonparametric form of MANOVA. Further, this procedure has the additional appeal that by creating random versions of $\hat{\boldsymbol{\beta}}_f$, random versions of $\bar{\mathbf{Y}}$are also created, meaning null distributions of trajectory attributes are generated from the same MANOVA steps.

It should be clear that computational steps only involve removing the interaction parameters, thus the analysis can accommodate any number of covariates (e.g., organismal size: see Adams and Collyer 2007). Further, statistical power limitations that are pervasive with parametric MANOVA (i.e., perhaps because of many evolutionary levels in the trajectories but few organisms to study) are less limiting with the residual randomization procedure.

Supporting Information

The following supporting information is available for this article:

Dataset. Phenotypic Trajectory Analysis.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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