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Phylogenetic Comparative Methods and the Evolution of Multivariate Phenotypes

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Abstract

Evolutionary biology is multivariate, and advances in phylogenetic comparative methods for multivariate phenotypes have surged to accommodate this fact. Evolutionary trends in multivariate phenotypes are derived from distances and directions between species in a multivariate phenotype space. For these patterns to be interpretable, phenotypes should be characterized by traits in commensurate units and scale. Visualizing such trends, as is achieved with phylomorphospaces, should continue to play a prominent role in macroevolutionary analyses. Evaluating phylogenetic generalized least squares (PGLS) models (e.g., phylogenetic analysis of variance and regression) is valuable, but using parametric procedures is limited to only a few phenotypic variables. In contrast, nonparametric, permutation-based PGLS methods provide a flexible alternative and are thus preferred for high-dimensional multivariate phenotypes. Permutation-based methods for evaluating covariation within multivariate phenotypes are also well established and can test evolutionary trends in phenotypic integration. However, comparing evolutionary rates and modes in multivariate phenotypes remains an important area of future development.

1. INTRODUCTION

Characterizing patterns of phenotypic diversity at macroevolutionary scales requires a phylogenetic perspective. It is widely recognized that shared evolutionary history leads to phenotypic similarity between closely related species, and thus statistical summaries must account for evolutionary nonindependence during the analysis (Felsenstein 1985, Harvey & Pagel 1991). The mathematical tools used to accomplish this task are known as phylogenetic comparative methods (Harmon 2018). Modern phylogenetic comparative biology began with the seminal work of Felsenstein (1985), whose phylogenetically independent contrasts revolutionized the way in which cross-species analyses are performed (for a recent review, see Huey et al. 2019). Subsequently, it was revealed that this approach is mathematically related to a broader class of statistical models [phylogenetic generalized least squares (PGLS): Grafen (1989), Martins & Hansen (1997)] (see Blomberg et al. 2012, Garland & Ives 2000, Rohlf 2001), thereby linking several approaches in one conceptual analytical framework. Thus, the current incarnation of the phylogenetic comparative toolkit was born.

Since these initial advances, there has been an explosion of analytical methods contributing to the phylogenetic comparative toolkit, enabling evolutionary biologists to quantify phenotypic trends that inform a wide array of biological hypotheses. For instance, phylogenetic comparative methods may be used to evaluate trends of evolutionary covariation between traits (Felsenstein 1985, Garland et al. 1993, Grafen 1989, Revell & Collar 2009), to quantify the degree of phylogenetic signal in phenotypes (Blomberg et al. 2003, Pagel 1999), to compare rates of phenotypic evolution among clades or between traits (Adams 2013, Garland 1992, O'Meara et al. 2006, Revell & Harmon 2008, Thomas et al. 2006), and to evaluate the fit of differing models of trait evolution (Beaulieu et al. 2012, Butler & King 2004, Hansen 1997). Unfortunately, while such approaches yield considerable power for characterizing patterns of phenotypic diversity across the tree of life, the biological insights derived from them have been largely restricted to univariate traits, as most comparative methods were developed for only a single column of phenotypic variables (e.g., body size). This is regrettable, as evolutionary biology is inherently multivariate (Blows 2007, Collyer et al. 2015), and processes such as natural selection can act on more than one trait simultaneously (Lande 1979, Lande & Arnold 1983). Furthermore, it has become common in evolutionary biology to characterize phenotypes using more than one trait (Harmon et al. 2008, Losos 1992, Price et al. 2010) or by using complex, multidimensional traits that require a vector of values to encode (Adams 2010, Kirkpatrick & Meyer 2004, McPeek et al. 2008). Thus, the ability to evaluate multivariate phenotypic trends across the phylogeny has become a pressing need.

Theorists have recently endeavored to develop phylogenetic comparative methods capable of evaluating phylogenetic patterns in multivariate data sets (e.g., Adams 2014b, 2014c; Adams & Collyer 2015; Bartoszek et al. 2012; Bastide et al. 2018; Goolsby 2015; Klingenberg & Marugán-Lobón 2013; Revell & Harmon 2008, among others). These methods are gaining prominence in the field and are increasingly used to address evolutionary hypotheses in multivariate phenotypic data sets in a manner analogous to what has long been possible for univariate traits (e.g., Chira et al. 2018, Felice & Goswami 2018, Grunstra et al. 2018, Martinez et al. 2018, Serb et al. 2017, Zelditch et al. 2015). In this review, we survey the recent advances for evaluating evolutionary trends in multivariate phenotypes, highlight some biological insights discovered using multivariate phylogenetic comparative approaches, and identify several areas for future analytical development. We describe the various types of data sets that biologists use to characterize multivariate phenotypes and relate these to the properties displayed by the resulting multivariate data spaces. We contend that visualizing patterns in multivariate phenotypic spaces plays an important role in macroevolutionary analyses and argue for the importance of such methods in complementing

quantitative macroevolutionary hypothesis testing. We then summarize multivariate phylogenetic hypothesis testing approaches, discuss their utility, and identify some current limitations for evaluating patterns in multivariate phenotypic data sets. Finally, we provide pertinent suggestions for empiricists to guide them in their analytical studies of multivariate phenotypes, as well as point to areas in need of future theoretical development.

2. CHARACTERIZING MULTIVARIATE PHENOTYPES

Before we summarize multivariate phylogenetic comparative methods, it is useful to review what is meant by a multivariate phenotype [note: evaluating evolutionary patterns in ecological data can also be performed multivariately (e.g., Pie et al. 2017) but is not discussed here]. Typically, a multivariate phenotype is a set of continuously measured trait values, which may be correlated with one another (Collyer & Adams 2007, Collyer et al. 2015, Huttegger & Mitteroecker 2011). Various data types are used to characterize multivariate phenotypes. For example, Catlett et al. (2010) measured gestation length, age at weaning, and other variables to represent multivariate life history phenotypes in lemurs. Patterns of multivariate gene expression have also been used (Valenzuela 2010). Likewise, sets of performance measures, including out-lever to in-lever ratios (Carroll et al. 2004), force and power estimates (Friedman et al. 2016), and empirical measures of locomotor performance (Moen et al. 2013), can represent multivariate phenotypes. Function-valued traits representing an ordered sequence of phenotypic values, such as data describing a growth curve, are also examples of multivariate phenotypes (Goolsby 2015, Kingsolver et al. 2001). However, most frequently, multivariate phenotypes describe morphological traits. Some common data types include sets of individual traits such as the lengths, ratios, and angles between structures [multivariate morphometrics: Blackith & Reyment (1971)], sets of shape variables obtained from the coordinates of anatomical points [landmark-based geometric morphometrics: Adams et al. (2013), Mitteroecker & Gunz (2009)], or variables derived from anatomical curves or surfaces, often obtained from computed tomography scans or other representations of anatomical objects [semilandmark methods: Gunz & Mitteroecker (2013); spherical harmonics: McPeek et al. (2008)]. **Figure 1** presents a visual summary of some common multivariate phenotypic data types.

Mathematically, multivariate phenotypes are represented by a vector of trait values. The vectors for a set of species are then assembled into an $N \times p$ matrix (**Y**), whose rows contain the phenotypes of the N species. The columns of **Y** contain the trait values for each of the p trait dimensions and correspond to the axes of a p-dimensional phenotypic data space. Therefore, each row of **Y** describes the location of a species as a point in this multivariate data space (**Figure 1**). The axes of the phenotypic data space are typically considered to be orthogonal, thereby assuming that the data space displays Euclidean geometry. This corresponds to the commonsense notion of data spaces where similar phenotypes are close together in the data space and dissimilar phenotypes are farther apart. Assuming that Euclidean geometry is appropriate for the span of phenotypic values in the data space also implies that distances and directions between specimens confer biological meaning, and comparisons of such measures are interpretable (see discussion in Huttegger & Mitteroecker 2011, Mitteroecker & Huttegger 2009). However, it is important to recognize that not all multivariate phenotypic data sets display these crucial properties.

For phenotypic data spaces to be Euclidean, at a minimum their trait dimensions must be in commensurate units and be of a similar scale. Otherwise, the mathematical definition of similarity and difference is not concordant across the trait dimensions (see Legendre & Legendre 2012). For example, a phenotypic data space could theoretically be constructed from a combination of continuous measurements, count variables (e.g., the number of scales), and the presence or absence of particular structures. However, relationships among specimens in this space are uninterpretable

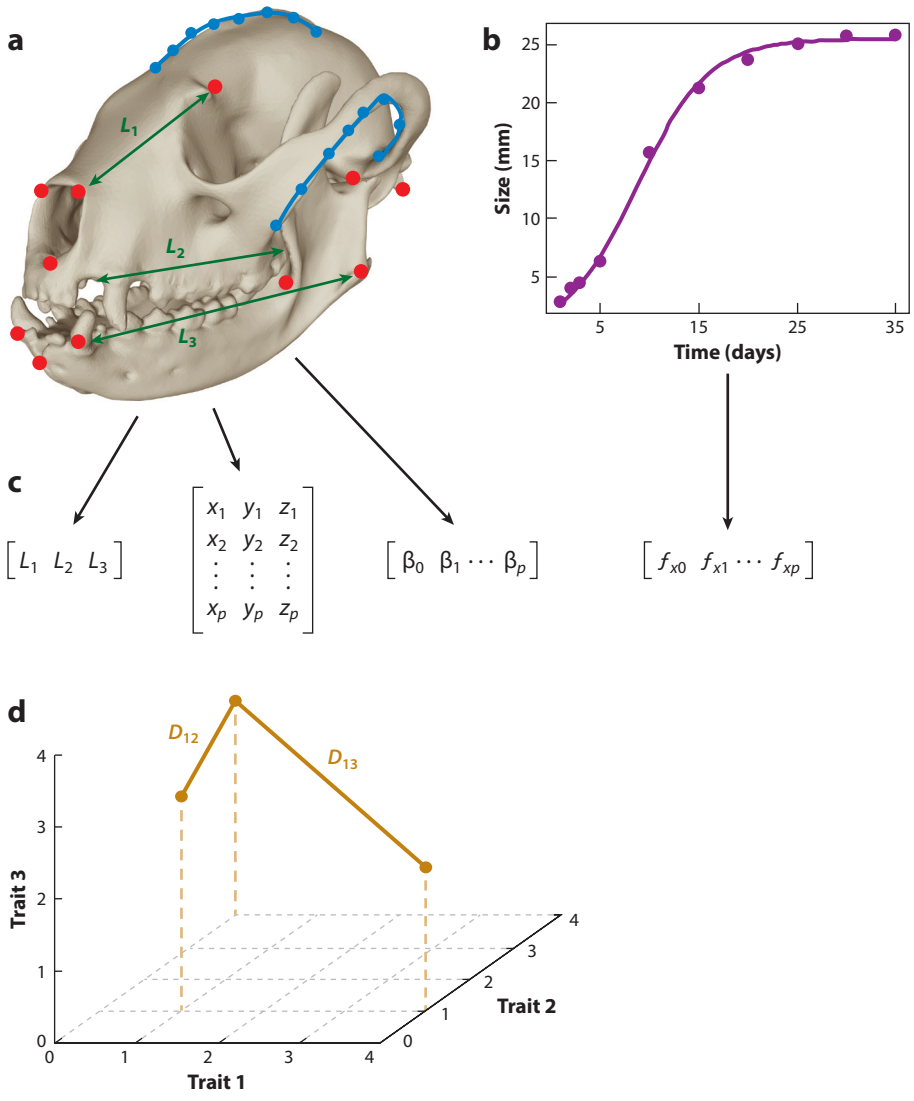

Figure 1

Examples of common multivariate phenotypic data. (*a*) Different types of measurements obtained from anatomical objects including linear distances (*green*), landmarks (*red*), and curves (*blue*). Panel *a* adapted from Phenome10K (**http://www.phenome10k.org**)/CC BY-NC 4.0; scan contributed to the database by Figueirido et al. (2014). (*b*) A function-valued trait representing a growth curve. (*c*) Multivariate phenotypes represented mathematically from each of the four data sets (*left to right*): linear distances, landmarks, curves, and function-valued traits. (*d*) Example of a multivariate phenotype space for a particular multivariate data set, with several species displayed. Numbers are hypothetical trait values. Species closer together in the space are similar in their multivariate phenotypes, while species further apart are less similar.

because the notion of distance differs between the axes representing continuous variables (Euclidean distance), counts (Gower's distance), and binary traits (Hamming distance). Importantly, this concern persists even for traits that are all continuously valued, if those traits are measured in different units (e.g., a data set of dimensionless ratios, masses, and angular extents). The reason is

that the deviations between specimens in each trait dimension are represented in differing units, and combining these deviations across traits to estimate distances between species, or covariances between traits, results in values that are uninterpretable. To construct a valid Euclidean space from such data first requires mathematical transformation or standardization of the trait dimensions, so the variables are expressed in similar units (see Legendre & Legendre 2012 for a related discussion). In such cases, however, some downstream phylogenetic comparative analyses may no longer be useful. For instance, estimates of disparity or evolutionary rates are not meaningful when performed on standard normal deviates, as standardizing data in this manner alters the original trait variances upon which disparity measures and evolutionary rates are based.

Fortunately, many phenotypic data sets are composed of variables measured in similar units and scale, thereby preserving these important properties. Examples include sets of linear measurements quantified in similar units and expressed in the same scale (but see Huttegger & Mitteroecker 2011), parameters summarizing function-valued traits or other curves, and shape variables from landmark-based geometric morphometric methods. In these cases, it is reasonable to assert Euclidean geometry, and empirical comparisons based on distances and directions in the multivariate phenotype space can be interpreted with respect to biological hypotheses. We recommend that empiricists carefully consider whether the phenotypic traits under investigation are of commensurate units and scale, so that downstream analyses of macroevolutionary patterns are interpretable.

Finally, multivariate phenotype spaces displaying Euclidean geometry also exhibit other useful properties, including rotation invariance. This means that the dispersion of species in the data space, and statistical summaries based on it, remain unchanged when the data space is viewed from a different orientation (see Adams & Collyer 2018a). Rigid rotations of data spaces are an essential component of many ordination methods, such as principal components analysis (PCA). As described in the next section, ordination methods are important tools for visualizing patterns of phenotypic evolution in multivariate phenotypes. And as explained below, rotation invariance is an essential property that multivariate phylogenetic comparative methods should retain.

3. VISUALIZING EVOLUTIONARY PATTERNS IN MULTIVARIATE PHENOTYPES

Because multivariate phenotypes are represented by many trait dimensions, visualizing patterns of phenotypic dispersion is often challenging. One solution is to use ordination methods that provide a low-dimensional view of a high-dimensional data space. Presently, two ordination methods incorporate phylogenetic information into their plots: phylomorphospaces and phylogenetic PCA.

3.1. Phylomorphospace

Phylomorphospaces are ordination plots with the phylogeny superimposed (Klingenberg & Ekau 1996, Rohlf 2002). For multivariate phenotypes, they provide a low-dimensional view of the phenotypic variation among the extant species while including hypothesized trait evolution along the branches of the phylogeny. To obtain a phylomorphospace, principal component (PC) axes are first obtained in the usual manner [i.e., from the $p \times p$ trait covariance matrix (**S**) calculated from **Y**]. Next, PC scores are obtained (via matrix projection) for all species, as well as for estimated ancestral values at the nodes of the phylogeny. Scores on the first few PC axes are then plotted to provide a graphical visualization of phenotypic dispersion relative to the phylogeny. Because this procedure is based on a decomposition of **S**, the axes of the phylomorphospace are orthogonal (Polly et al. 2013), and the method is thus a rigid rotation of the original phenotype space.

Additionally, scores on the PC axes are uncorrelated with one another, and patterns of dispersion among species are retained. This means that the approach preserves the total variation in the data set throughout the analysis and that distances and directions among species may be interpreted biologically. Note, however, that the phylomorphospace plot is a projection of the full data space into a subspace of fewer dimensions (typically two). Thus, exploring patterns in higher dimensions may yield additional revelations. Additionally, any downstream statistical analyses should be performed on the full set of trait dimensions to ensure that 100% of the phenotypic variation is included in the analysis. When subsets of trait dimensions are evaluated (e.g., the first few PCs), evolutionary inferences from them can be misleading (Adams & Collyer 2018a, Uyeda et al. 2015; for a related discussion, see Bookstein 2013).

Phylomorphospaces provide powerful tools for visualizing evolutionary patterns in multivariate phenotypes, which can lead to significant biological insights. For example, Aristide et al. (2018) discovered that New World monkeys diversified into distinct regions of morphospace with little phenotypic overlap among genera, a pattern interpreted as being consistent with an adaptive radiation. Likewise, Davis & Betancur-R (2017) found that herbivorous and carnivorous fish species occupied distinct regions of morphospace, and did so consistently across lineages, demonstrating strong phylogenetic convergence of ecotypes in the group. In other cases, phylomorphospaces have revealed that one lineage displays considerably greater phenotypic disparity as compared to another lineage (e.g., Sidlauskas 2008, Zelditch et al. 2015), implying possible selective release, differing rates of morphological evolution between lineages, or that one lineage repeatedly evolves similar phenotypes throughout its evolutionary history. Finally, phylomorphospaces can be used to identify groups where phenotypes appear accentuated in a consistent manner over time (Sherratt et al. 2016), providing evidence of a directional trend in multivariate phenotypic evolution. Several hypothetical examples of patterns commonly observed in phylomorphospaces are shown in **Figure 2**.

Importantly, phylomorphospaces provide a visual means of examining evolutionary patterns that may be evaluated quantitatively using statistical hypothesis testing approaches. For example, visual patterns of phenotypic distinctness among ecotypes may be formally evaluated using phylogenetic analysis of variance (ANOVA) (sensu Adams & Collyer 2018b), while patterns of recurrent phenotypic evolution may be confirmed via statistical tests of evolutionary convergence (see Stayton 2015). Likewise, apparent differences in phenotypic variance may be evaluated formally via disparity comparisons among clades (Serb et al. 2017, Zelditch et al. 2015) and by comparisons of rates of phenotypic evolution (Sherratt et al. 2017). Finally, patterns revealed in phylomorphospaces may precipitate simulation-based approaches to explore more complex macroevolutionary hypotheses and evolutionary scenarios (e.g., Sherratt et al. 2016, Sidlauskas 2008). We assert that phylomorphospaces are an important component of the multivariate phylogenetic comparative toolkit and recommend that empiricists employ them as a regular part of their statistical arsenal for evaluating trends in multivariate phenotypes.

3.2. Phylogenetic Principal Components Analysis

Phylogenetic principal components analysis (pPCA) (Revell 2009) is another approach for obtaining ordination plots while accounting for phylogenetic nonindependence. Statistically, the method conditions the ordination on the phylogeny via a decomposition of the $p \times p$ evolutionary rate matrix (**R**) (Revell & Harmon 2008) rather than using the original **S**. The difference between the two is that **R** is simply **S** standardized by the phylogeny and is therefore weighted inversely by the evolutionary relationships among taxa. PC scores are then obtained for the extant species as well as for the estimated ancestral taxa, and these scores are used to generate the ordination plot (for details, see Revell 2009).

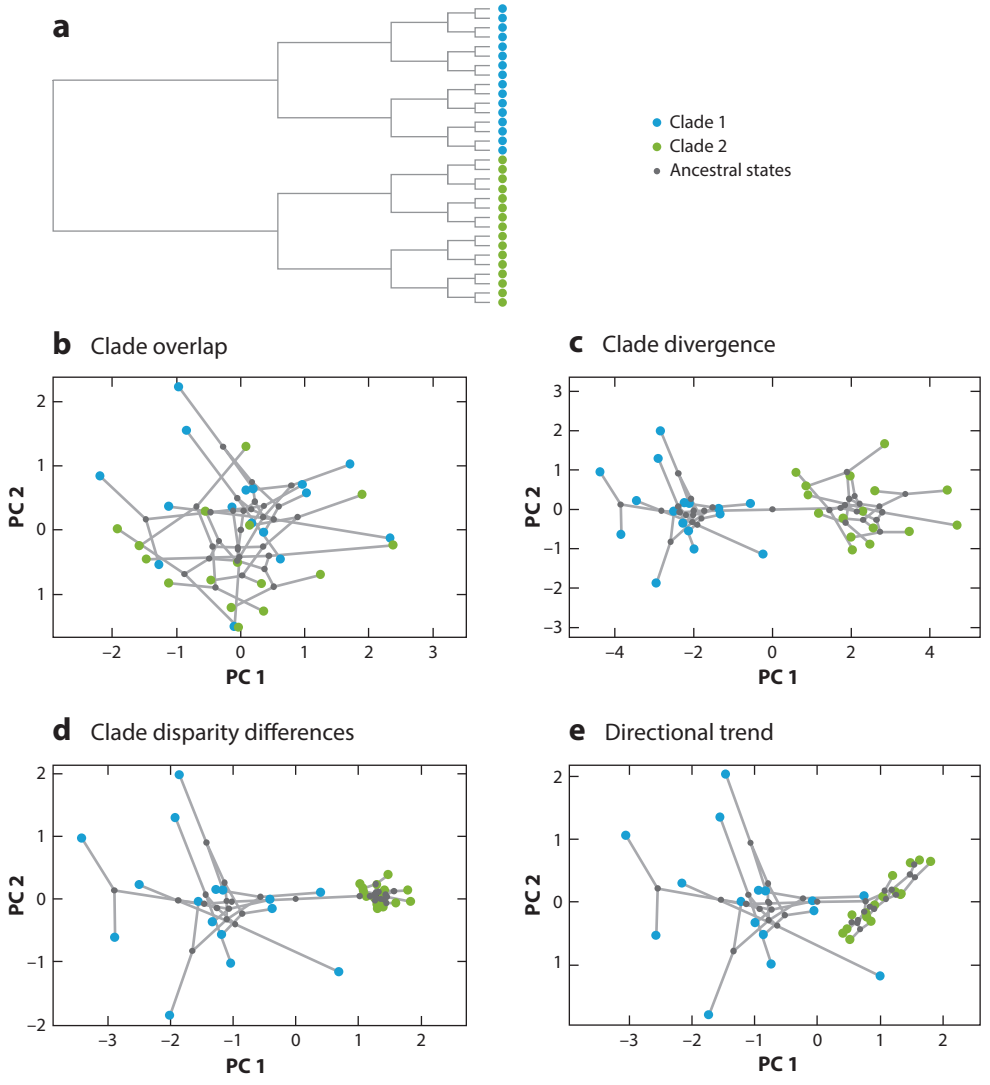

Figure 2

Hypothetical examples of phylomorphospace plots for 32 species related by a phylogeny. (*a*) Clades for these species are denoted in blue and green. Phylomorphospace patterns include (*b*) clade overlap in multivariate phenotype space, (*c*) clade divergence of phenotypes, (*d*) differences in clade disparity, and (*e*) directional phenotypic evolution in the green clade. Abbreviation: PC, principal component.

While phylogenetic PCA has the appeal of incorporating the phylogeny into its computations, the approach does have some nonintuitive properties. For instance, the axes of phylogenetic PCA are orthogonal, yet the species' scores on those axes are correlated with one another (see also Polly et al. 2013). And unlike standard PCA, the eigenvalues of the phylogenetically corrected PC axes do not sum to the total phenotypic variation in the data set [although summing variation in the phylogenetic PC scores does: Polly et al. (2013)]. Additionally, the statistical rationale for incorporating the phylogeny into the computations is unclear, as PCA does not assume independence among observations, in contrast to hypothesis testing approaches that often require this

assumption (e.g., ordinary least squares ANOVA and regression methods: see Section 5.2 below). However, the axes of phylogenetic PCA have been rotated to account for the effects of phylogeny, and when all axes are used, the distances between species in the phylogenetically rotated space are identical to that of the original multivariate data space. Overall we agree with Polly et al. (2013) that results from phylogenetic PCA can be difficult to interpret and instead recommend phylomorphospaces as a means of visualizing dispersion in multivariate phenotypes. Nonetheless, we acknowledge that obtaining a visualization of multivariate phenotype spaces that aligns with the phylogenetic relatedness among species is an important goal worth pursuing. We therefore recommend that future theoretical work should explore alternative algebraic formulations to produce ordinations that maximize the covariation between the phenotypic data and the phylogeny but do so while preserving the desirable properties displayed by classical ordination approaches.

4. PHYLOGENETIC SIGNAL IN MULTIVARIATE PHENOTYPES

Phylogenetic signal is the tendency for related species to be more phenotypically similar than species selected at random from a phylogeny (Blomberg et al. 2003, Munkemuller et al. 2012). Phylogenetic signal is expected under many macroevolutionary scenarios of trait evolution and is therefore frequently examined in phylogenetic comparative studies. For multivariate phenotypes, several analytical approaches are available. One method (Pagel 1999) evaluates the fit of the data to the phylogeny while including a scaling parameter (λ) that describes the degree of phylogenetic signal, but this approach is limited to cases where the number of variables is less than the number of species (see Adams 2014a). Another approach quantifies the sum of squared changes in multivariate phenotypes across branches of the phylogeny (Klingenberg & Gidaszewski 2010). However, this method is sensitive to both the number of variables (p) and the number of species (N), complicating comparisons across data sets (Adams 2014a). Additionally, because this approach is based on ancestral state estimation, it provides inaccurate estimates of phylogenetic signal when directional phenotypic evolution has occurred, as ancestral states are not faithfully estimated under scenarios of directional evolution (Royer-Carenzi & Didier 2016). A third approach (Adams 2014a) is a multivariate generalization of the kappa statistic (Blomberg et al. 2003) that measures phylogenetic signal as a ratio of observed to expected phenotypic variation obtained with and without considering phylogenetic nonindependence. This approach (K_{mult}) has a known and constant expected value, per variable, under Brownian motion (1.0) and holds considerable promise for characterizing the degree of phylogenetic signal in multivariate data sets.

To provide a sense of the degree to which multivariate phenotypes display phylogenetic signal, we surveyed the literature for empirical studies of phylogenetic signal in multivariate phenotypes, obtaining over 330 K_{mult} estimates from nearly 100 published studies. The vast majority of these data sets (80%) described morphological phenotypes, while the remainder represented multivariate life history traits, behavior, physiology, and other measures. Overall, the degree of phylogenetic signal was not significantly different among these data types ($R^2 = 0.038$; $F = 1.867$, $P = 0.09$), although behavioral data did exhibit lower values as compared to the other phenotypic data sets ($K_{mult} = 0.47$ versus $K_{mult} = 0.65$). K_{mult} ranged from 0.031 to 2.130 in this sample (**Figure 3**), with a mean phylogenetic signal of 0.65. K_{mult} did not vary with the number of species in the phylogeny ($R^2 = 0.002$; $F = 0.7557$, $P = 0.368$), and we observed that most of the values were less than 1.0. Interestingly, all these findings were generally consistent with patterns observed in an earlier survey of phylogenetic signal in univariate phenotypes (Blomberg et al. 2003). This suggests that broadscale patterns of phylogenetic signal may be concordant across both single-valued and multivariate phenotypes. Additionally, approximately 75% of the K_{mult} values displayed significant phylogenetic signal, although there was no difference in the distributions of K_{mult} for significant

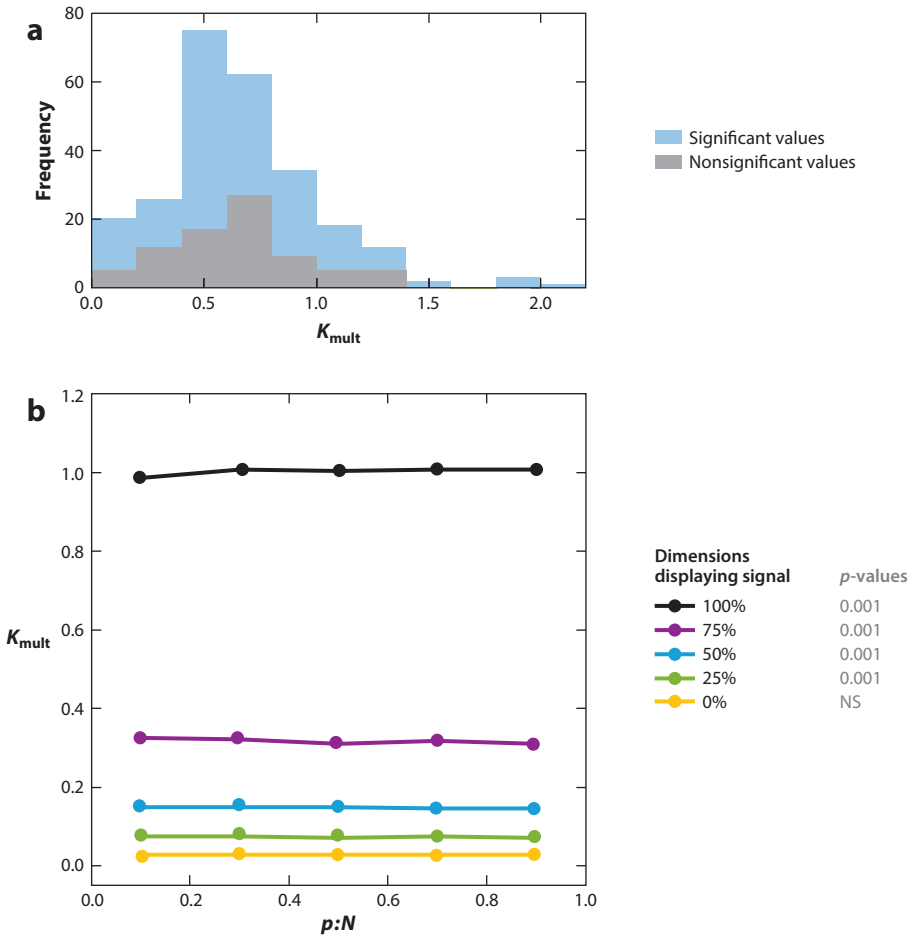

Figure 3

Patterns of phylogenetic signal in multivariate phenotypes. (*a*) Frequency distribution of the degree of phylogenetic signal in multivariate phenotypes from 330 empirical data sets obtained from the literature. (*b*) Phylogenetic signal represented as the mean across 5 multivariate data sets simulated under Brownian motion evolution across a phylogeny of 400 species ($N = 400$). At each simulation level, the ratio of variables to species (*p:N*) was altered, and the number of trait dimensions simulated under Brownian motion, versus the number of dimensions whose traits were generated with no association to the phylogeny, was altered. Estimates of phylogenetic signal (K_{mult}) and of statistical significance are shown.

and nonsignificant data sets ($t = 0.5498$, $P = 0.5832$; $D_{KS.test} = 0.0698$, $P = 0.9251$). This paradoxical result makes sense considering phylogenetic signal latency, which we discuss below.

One interesting observation in this sample was that most estimates of multivariate phylogenetic signal were considerably less than 1.0, indicating that there was less phylogenetic signal than expected under Brownian motion. Yet the majority of these data sets displayed statistically significant phylogenetic signal when compared to a random association of phenotypes to the tips of the phylogeny. Indeed, a similar pattern was also observed in univariate data sets (Blomberg et al. 2003), where it was attributed to either selection and phenotypic adaptation (which would reduce variation across taxa) or to various sources of measurement error. While such explanations remain a possibility here, for multivariate phenotypes we suggest a third possibility. Specifically,

when phylogenetic signal is concentrated in one or a few phenotypic dimensions, it is possible to observe significant multivariate phylogenetic signal whose summary measure (K_{mult}) is less than 1.0. This possibility was explored briefly through a simulation study in which a subset of trait dimensions was obtained via phylogenetic simulation under Brownian motion, while the remaining trait dimensions contained variation that was not phylogenetically associated. Results of these simulations (**Figure 3**) confirmed that as the percentage of trait dimensions with nonphylogenetically associated variation increases, K_{mult} decreases (but the ratio of p to N does not impact K_{mult}). Yet because there was still phylogenetic signal in some trait dimensions, many such data sets still display significant phylogenetic signal. Biologists who observe this pattern in their empirical data sets should consider whether phylogenetic signal is concentrated in a subset of trait dimensions in their data as a possible explanation of this pattern. Unfortunately, it is currently not straightforward how to discern between a weak signal for many variables and a strong signal for few variables for small values of K_{mult}, other than to consider the significance of the signal based on the p-value. We propose that future research should consider evaluations of the effect size of K_{mult}, calculated as a standard deviate from its sampling distribution [values produced from resampling permutations: see related statistics derived in Adams & Collyer (2016, 2018b), Collyer et al. (2015)], as a way to resolve this conundrum.

Finally, for both univariate and multivariate phenotypes, current statistical tests do not evaluate whether the observed pattern differs from what is expected under Brownian motion. Instead, they evaluate the observed phylogenetic signal relative to a sampling distribution obtained by permuting phenotypic values across the tips of the phylogeny (i.e., a random association of phenotypes with species) (for the conceptual motivation of the original test, see Blomberg et al. 2003). To fill this void, we propose an additional approach where the observed K_{mult} is compared to a distribution of values obtained from data simulated on the phylogeny under Brownian motion. This simulated distribution will have an expected value of 1.0 (see Adams 2014a), and thus comparisons to this distribution will evaluate whether the observed phylogenetic signal differs from what is expected under Brownian motion. Future theoretical work should formally develop the test procedure proposed here and evaluate its statistical properties.

5. MULTIVARIATE PHENOTYPES AND PATTERNS OF COVARIATION

For many evolutionary hypotheses, understanding the degree to which phenotypic traits covary is of paramount importance. There are many biological reasons to expect that multivariate phenotypes would display correlations. For instance, common selective pressures and other mechanisms can generate covariation between phenotypic traits within an organism (Klingenberg 2014). Likewise, selection and adaptation can generate correlations between phenotypes and other parameters such as diet, climate, the presence or absence of competing species, or other ecological variables (e.g., Baab et al. 2014, Mahler et al. 2010, Martin & Wainwright 2011). Several analytical methods have been developed for evaluating patterns of trait covariation in multivariate phenotypes. Determining which method should be used depends in part upon whether the evolutionary correlations of interest are between phenotypic traits or whether they describe the evolutionary covariation between phenotypes and other variables. Below we highlight methods for evaluating both types of patterns and identify several biological hypotheses that can be addressed with these approaches.

5.1. Phenotypic Integration: Evolutionary Correlations Within Phenotypes

Phenotypic integration describes a pattern where phenotypic traits are correlated with one another (Olson & Miller 1958). Such patterns are expected when selection acts upon multiple

functionally related traits (Arnold 2005) or when traits display genetic linkages, exhibit pleiotropy, or have shared developmental pathways (see Cheverud 1996, Mitteroecker & Bookstein 2007). Several analytical methods have been developed to evaluate evolutionary correlations among traits in a phylogenetic context. For example, Revell & Collar (2009) used a likelihood framework to evaluate changes in evolutionary correlations between traits across the phylogeny. For instance, this method revealed that suction-feeding eel species exhibited higher evolutionary correlations between anatomical units as compared to bite-feeding species (Collar et al. 2014). Similarly, a Bayesian approach can be used to evaluate shifts in evolutionary correlations between traits across the phylogeny (Caetano & Harmon 2019). Both methods have potential to yield insights that may inform on how evolutionary correlations evolve. Nonetheless, it should be recognized that they consider only pairwise correlations between individual traits because both methods evaluate the elements of **R**, which contains pairwise evolutionary trait correlations. When broader patterns of evolutionary correlations across traits are of interest, other analytical approaches are required.

Sometimes it is of interest to determine whether sets of traits (or modules) display evolutionary correlations with one another. For example, one may wish to determine whether distinct anatomical modules, such as the skull and mandible, correlate across the phylogeny (e.g., Adams & Felice 2014, Figueirido et al. 2010). To evaluate such patterns, a multivariate equivalent of evolutionary correlation is required. Here, phylogenetic partial least squares (Adams & Felice 2014, Klingenberg & Marugán-Lobón 2013) may be used to evaluate covariation in multivariate phenotypes. As with pairwise evolutionary correlations, the approach starts with **R** (see above). However, rather than evaluating each evolutionary correlation individually, the overall covariation between blocks of variables is quantified and is evaluated. In one example, high levels of evolutionary integration between the basicranium and facial regions of primates were identified, a pattern that has impacted the evolution of phenotypic disparity in this group (Neaux et al. 2018). In another study, Evans et al. (2017) found that phenotypic integration between the braincase and facial regions of some teleost fishes was greater than that exhibited in carnivore mammals and that the lower levels of integration in carnivores allowed for greater opportunity to evolve phenotypic disparity. Similarly, phenotypic integration between claw and toepad traits in *Anolis* lizards was found to enhance microhabitat specialization among species (Yuan et al. 2019). These and other examples are a testament to the power of phylogenetic partial least squares for identifying evolutionary correlations between sets of variables.

Finally, it may be of interest to determine whether all traits in a multivariate data set display phenotypic integration with one another. Unfortunately, methods for evaluating such patterns in a phylogenetic context are less well developed. For patterns across individuals within species, summary measures such as eigenvalue variance may be used to characterize patterns of global integration (e.g., Pavlicev et al. 2009; for an alternative approach, see Bookstein 2015). However, while the phylogenetic distribution of variation across PC dimensions has been visually examined (e.g., Klingenberg & Marugán-Lobón 2013), to our knowledge neither a phylogenetic equivalent of the above summary measures nor a statistical test of such measures has been proposed. We recommend that future theoretical work investigate this possibility and develop formal statistical tests of global integration in a phylogenetic context.

5.2. Phylogenetic Linear Models: Analysis of Variance and Regression

Many evolutionary hypotheses strive to evaluate the relationship between multivariate phenotypes and one or more independent variables. Such hypotheses are best characterized by PGLS models

(Grafen 1989, Martins & Hansen 1997). These models are defined mathematically as

$$\mathbf{Y} = \mathbf{XB} + \mathbf{E}, \quad 1.$$

where $\mathbf{Y}$ is an $N \times p$ matrix of multivariate phenotypic trait values, $\mathbf{X}$ is an $N \times k$ design matrix containing one or more independent (predictor) variables, $\mathbf{B}$ is a $k \times p$ matrix containing the model coefficients, and $\mathbf{E}$ is an $N \times p$ matrix of residuals (see Adams 2014b; Adams & Collyer 2015, 2018b; Clavel et al. 2015). Unlike ordinary least squares models where the residual error ($\mathbf{E}$) is assumed to be independent, the residuals of PGLS are not independent but instead contain the expected covariation between species as described by the phylogenetic covariance matrix under a specified model of evolutionary change [typically Brownian motion: Rohlf (2001)]. Thus, the analysis is tantamount to a weighted least squares model, where the weights are the inverse of phylogenetic relatedness.

Implementing PGLS models using parametric statistical techniques based on maximum likelihood and other formulations has long been the favored approach for evaluating evolutionary trends in univariate data (e.g., Grafen 1989, Martins & Hansen 1997). However, multivariate phenotypes present numerous challenges to this paradigm that have only recently come to light (see Adams 2014b, Adams & Collyer 2018a). For instance, approaches that evaluate multivariate PGLS models using maximum likelihood, or through standard multivariate test measures (e.g., Wilks's Λ), display increasing type I errors as phenotypes become more highly multivariate (Adams 2014b, Adams & Collyer 2018a). The reason is that standard parametric implementations require finding the determinant and the inverse of the $p \times p$ trait covariance matrix, which becomes more challenging as the number of traits (p) approaches the number of species (N) and is not possible when $p > N$ (see Adams 2014b; Adams & Collyer 2018a, 2018b). To circumvent these issues, pairwise composite likelihood measures combined with phylogenetic simulations were proposed (Goolsby 2016). However, this method is not rotation invariant, and statistical conclusions from it differ for the same phenotypic data set when viewed in different orientations (for additional issues, see Adams & Collyer 2018a). This observation emphasizes why properties such as rotation invariance are important in multivariate comparative analyses and demonstrates that analytical methods for describing evolutionary patterns in multivariate phenotypes should retain this important property if they are to provide useful biological inferences.

An alternative implementation to multivariate PGLS uses phylogenetic transformation and test statistics derived from traces of covariance matrices rather than determinants, making them more robust to the challenges described above (Adams 2014b; Adams & Collyer 2015, 2018b). Statistical evaluation of these measures is then accomplished using residual randomization permutation procedures (Adams & Collyer 2018b, Collyer & Adams 2018), where residuals from a reduced model are permuted to generate empirical sampling distributions against which the observed test statistics are compared. This approach is rotation invariant, yields identical model parameters to standard implementations, and displays appropriate type I errors and high statistical power; sampling distributions generated from it align with statistical distributions derived from statistical theory (see Adams & Collyer 2018a,b). Finally, the method can be used for various statistical designs, including phylogenetic ANOVA, phylogenetic regression, phylogenetic factorial models, and phylogenetic analyses of covariance.

An increasing number of empirical studies use permutation-based PGLS to evaluate patterns in multivariate phenotypes. For example, Paluh & Bauer (2018) examined the evolution of quadrate shape in geckos, revealing distinct allometric trajectories across genera, suggesting that disparate functional pressures resulted in shifts in the direction of evolutionary allometry among lineages. Likewise, patterns of jaw shape variation were found to covary with dietary preferences in both

tree-dwelling and ground-dwelling squirrel species (Zelditch et al. 2017). In fact, ecomorphological associations between microhabitat use and multivariate phenotypes have been identified in numerous lineages using this approach, including butterflies (Chazot et al. 2015), lacertid lizards (Hipsley & Muller 2017), marine scallops (Serb et al. 2017, Sherratt et al. 2016), and other taxa. In general, permutation-based PGLS approaches provide a powerful tool for understanding patterns of covariation in multivariate phenotypes. We recommend that permutation-based PGLS be used in future studies to evaluate covariation in multivariate phenotypes as described by phylogenetic linear models (regression, ANOVA, etc.).

6. EVOLUTIONARY TEMPO AND MODE IN MULTIVARIATE PHENOTYPES

Phylogenetic comparative methods describe the accumulation of phenotypic variation across the phylogeny under some process of evolutionary change. Typically, Brownian motion is used, where trait variation accumulates proportional to time under random (neutral) trait perturbations (Felsenstein 1973, 1981). However, other models of evolutionary change could be envisioned, such as Ornstein-Uhlenbeck (OU) models that incorporate selection into the variance-generating process (Butler & King 2004, Hansen 1997). Recent years have seen the development of analytical methods for comparing the fit of phenotypic data to the phylogeny under alternative evolutionary models. These methods have the advantage of statistically comparing different evolutionary scenarios by evaluating the fit of the data to the phylogeny under different models for hypothesized processes (e.g., Beaulieu et al. 2012, Butler & King 2004). Typically, such evolutionary model fitting approaches are described from a likelihood perspective; however, it is important to recognize that algebraically these methods can also be described using the PGLS model:

$$\mathbf{Y} = 1\mathbf{B} + \mathbf{E}, \qquad 2.$$

where $\mathbf{Y}$ is the matrix of multivariate phenotypic trait values, 1 is a column of ones (indicating a single-mean model), $\mathbf{B}$ is a vector of model coefficients, and $\mathbf{E}$ is a matrix of residuals. In this case, the residuals of the model are normally distributed but only under the specific model of evolutionary change (e.g., Brownian motion, OU, etc.). Viewed from this framework, different evolutionary models can be described by using different evolutionary covariance matrices embodied by $\mathbf{E}$ (see Adams & Collyer 2018a, Clavel et al. 2015). Thus, evolutionary model comparisons are accomplished by obtaining summary statistics [e.g., logL or Akaike information criterion (AIC)] describing the fit of the data to the phylogeny under differing models of trait evolution and selecting the preferred model based on these statistics (e.g., Butler & King 2004). While most analytical methods for comparing evolutionary models were developed for univariate traits (Beaulieu et al. 2012, Butler & King 2004, O'Meara et al. 2006, Thomas et al. 2006), several approaches can now accommodate model comparisons for multivariate phenotypes.

6.1. The Tempo of Evolution in Multivariate Phenotypes

One class of models facilitates comparisons of rates of phenotypic evolution across lineages. Here the fit of the data to the phylogeny is obtained under a model containing a single rate of evolutionary change for all species and then under a second model where rates of evolution differ between two or more groups. For multivariate phenotypes, this is tantamount to fitting the data to the phylogeny using one or more evolutionary rate matrices, where the evolutionary rates (σ^2) for each phenotypic trait dimension are found along the diagonal of the $p \times p$ of $\mathbf{R}$. One approach

compares the fit of one or more evolutionary rate matrices to the phylogeny using likelihood ratio tests and AIC values (Clavel et al. 2015, Revell & Harmon 2008) (for a related Bayesian approach, see Caetano & Harmon 2019). Such likelihood methods can be appropriate when one is evaluating rates of phenotypic evolution from many taxa and just a few phenotypic trait variables (i.e., when $N \gg p$) (see Revell & Harmon 2008). However, when the $N{:}p$ ratio decreases, these methods suffer from high levels of model misspecification favoring multirate models when data were generated under a single-rate model. Additionally, as the phenotypic data become more highly multivariate, model misspecification errors increase precipitously (Adams 2014c, Adams & Collyer 2018a). Therefore, likelihood-based methods for evaluating shifts in evolutionary rate matrices are not a general solution for evaluating rate shifts in high-dimensional phenotypic data and are appropriate only when the data set comprises a few trait dimensions and many species (i.e., when $N \gg p$).

An alternative approach estimates a single multivariate rate of evolution (σ^2_{mult}) for all traits simultaneously (Adams 2014c) and uses simulations or permutations to evaluate the fit of the data under a single-rate versus a multirate model. Similarly, evolutionary rates among several multivariate phenotypes for the same taxa can be compared using σ^2_{mult} (see Denton & Adams 2015). Both approaches are robust to the challenges described above, and tests based on them display appropriate statistical properties. For instance, rates of evolution were found to differ in distinct regions of the avian skull, and these patterns were negatively correlated with levels of integration within modules (Felice & Goswami 2018). A similar pattern was discovered in ray-finned fishes, where lower levels of modularity in some traits were suggested to promote phenotypic diversification (Larouche et al. 2018). Rates of multivariate phenotypic evolution have also been shown to differ in shell shapes among ecotypes of marine scallops (Sherratt et al. 2017), in the pectoral fins of acanthomorph fishes (Du et al. 2019), and in body shape evolution between several endangered freshwater fish lineages (Foster & Piller 2018), among other examples.

One limitation with these methods is that σ^2_{mult} describes the net evolutionary rate of change across the entire multivariate phenotype space. Therefore, if rates of evolution along individual trait axes are of interest, methods more akin to those developed using **R** would be appropriate, although current implementations display high rates of model misspecification when phenotypes are highly multivariate. We consider having robust methods based on **R** for high-dimensional phenotypes as a current analytical need and thus recommend that future theoretical work focus on the development of robust methods that can evaluate sets of phenotypic traits individually but do so in a manner that minimizes misspecification rates and maximizes statistical power.

6.2. Multivariate Phenotypes and Evolutionary Mode

Because Brownian motion may not be the most appropriate model for describing patterns of phenotypic evolution, models that incorporate selection and other evolutionary processes have been developed. For instance, likelihood-based analytical approaches can characterize selection-based models in multivariate phenotypes, such as those embodied by OU processes. One method attempts to discover how many adaptive peaks are observed in a multivariate data set by fitting a series of Brownian motion and OU models to each trait dimension separately and combining the optimal fitting models across trait dimensions to arrive at an estimate of the adaptive landscape for the data set (Ingram & Mahler 2013). Unfortunately, the method suffers from extremely high levels of model misspecification and is unreliable. In a recent analysis, nearly 95% of data sets simulated under Brownian motion were incorrectly predicted to display two or more OU-adaptive peaks (see Adams & Collyer 2018a). Further, comparing predicted patterns from the observed data set to those obtained from data simulated under Brownian motion does not provide additional insight. With this approach, one could, for instance, determine that there were more

predicted adaptive peaks in the observed data set than in the simulated data. However, even under such circumstances, it is still unknown which of the predicted adaptive peaks represent the real peaks in the data (if any) and which describe the peaks generated by the method. Thus, while the method has considerable intuitive appeal, its current implementation is prohibitive for biological insight (for additional issues with the approach, see Adams & Collyer 2018a).

Several other approaches have been developed that fit OU models to multivariate phenotypic data (e.g., Bartoszek et al. 2012, Bastide et al. 2018, Clavel et al. 2015, Goolsby 2016, Khabbazian et al. 2016). With these methods, one fits the data to a series of a priori models (Brownian motion, OU 1-peak, OU 2-peaks, etc.), and summary measures such as AIC are used to identify the model with the highest support. However, at present, all current implementations of multivariate OU models suffer from at least one of three critical shortcomings (see Adams & Collyer 2018a). Either the methods display very high levels of model misspecification (preferring more complex models for data simulated under Brownian motion) that increase with trait dimensionality, or they are not rotation invariant (meaning different outcomes are obtained for the same data viewed in different orientations), or they work only if data dimensionality is reduced to accommodate log-likelihood estimation. One reason for these issues is the large number of parameters that must be estimated (particularly covariance terms) even for relatively simple evolutionary models and just a few trait dimensions. For instance, whereas a two-peak OU model for univariate data is described by 4 parameters (1 alpha, 1 sigma, and 2 theta parameters), the same model for three-dimensional data requires up to 18 parameters (6 alpha, 6 sigma, and 6 theta parameters). Likewise, a three-group rate shift model for three phenotypic trait dimensions requires up to 21 parameters to encode (18 sigma and 3 phylogenetic mean parameters). Clearly, evolutionary models can become very parameter rich, even for multivariate phenotypes described by only a few trait dimensions. Thus, comparing complex evolutionary models, even in those cases where $N \gg p$, is still problematic because of the large number of parameters that must be accurately estimated and evaluated. As such, at present we lack a reliable approach for fitting OU models to multivariate phenotypes, which is clearly a pressing need. Future theoretical work should explore alternative implementations to arrive at a robust approach to evaluating multivariate OU models.

7. CONCLUSIONS

The past decade has seen tremendous growth in the use of phylogenetic comparative methods to evaluate evolutionary trends in multivariate phenotypes. Methods for visualizing such trends in high-dimensional data sets are now available, the degree of phylogenetic signal can be reliably quantified, and patterns of covariation—both within multivariate phenotypes and between multivariate phenotypes and other variables—can be characterized. We identified several current analytical challenges in the field where methods need to be developed and remain confident that the scientific community is up to the task. As the field continues to mature, we are excited for the future discoveries empiricists will make that will enrich our understanding of how evolutionary processes shape patterns of diversity in multivariate phenotypes across the tree of life.

SUMMARY POINTS

1. Phylogenetic comparative analyses on multivariate phenotypes describe patterns of dispersion in multivariate data spaces. For these analyses to have meaning, distances and directions in the phenotype space must be interpretable. To ensure this, the axes of multivariate phenotypes should be in commensurate units and scale. Empiricists should

carefully consider whether the phenotypic traits they use are in commensurate units and scale, so that downstream analyses of macroevolutionary patterns are interpretable.

2. Phylomorphospaces provide a low-dimensional visualization of a high-dimensional phenotypic data space. They are extremely useful for describing multivariate trait evolution and for generating hypotheses for future evaluation. Phylomorphospaces should be used as a regular part of any macroevolutionary analysis of multivariate phenotypes.
3. Phylogenetic signal in multivariate phenotypes is quantified in a manner analogous to what is accomplished for univariate traits. Patterns of phylogenetic signal in different types of multivariate phenotypes are similar to those observed in univariate traits. Most data sets display significant phylogenetic signal, yet those values are less than what is expected under Brownian motion.
4. Testing hypotheses of covariation in multivariate phenotypes becomes challenging as the number of trait dimensions increases. For this reason, standard (parametric) statistical hypothesis testing methods break down as phenotypes become more highly multivariate. Phylogenetic transformation, combined with the use of robust summary statistics and permutation methods (residual randomization), provides a solution to these challenges so that patterns described by linear models can be evaluated in a phylogenetic framework. Permutation-based phylogenetic generalized least squares should be a regular part of the macroevolutionary toolkit for evaluating hypotheses of covariation in multivariate phenotypes while accounting for phylogenetic nonindependence.
5. Characterizing the way phenotypic diversity accumulates is accomplished by evaluating alternative evolutionary models. For multivariate phenotypes, the net rate of evolutionary change under Brownian motion can be reliably compared between clades and between multivariate traits. However, characterizing non-Brownian evolution in multivariate phenotypes, such as processes described by Ornstein-Uhlenbeck models, represents a current challenge in the field.

FUTURE ISSUES

1. New phylogenetic ordination approaches that maximize the covariation between the phenotypic data and the phylogeny should be developed.
2. Tests evaluating phylogenetic signal relative to what is expected under Brownian motion or other evolutionary models should be formalized for both univariate and multivariate phenotypes.
3. Robust evolutionary models for describing trait change in highly multivariate phenotypes, including Ornstein-Uhlenbeck models, are a critical need and should be developed.

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