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TOOLS AND TECHNIQUES

Searching for Modular Structure in Complex Phenotypes: Inferences from Network Theory

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Abstract The notion of modularity has become a unifying principle to understand structural and functional aspects of biological organization at different levels of complexity. Recently, deciphering the modular organization of molecular systems has been greatly aided by network theory. Nevertheless, network theory is completely absent from the investigation of modularity of complex macroscopic phenotypes, a fundamental level of organization at which organisms experience and interact with the environment. Here, we used geometric descriptors of phenotypic variation to derive a network representation of a complex morphological structure, the mammalian mandible, in terms of nodes and links. Then, by integrating the network representation and description with random matrix theory, we uncovered a modular organization for the mammalian mandible, which deviates significantly from an equivalent random network. The modules revealed by the network analysis correspond to the four morphogenetic units recognized for the mammalian mandible on a developmental basis. Furthermore, these modules are known to be affected only by particular genes and are also functionally differentiated. This study shows that the powerful formalism of network theory can be applied to the discovery of modules in complex phenotypes and opens the possibility of an integrated approach to the study of modularity at all levels of organizational complexity.

Keywords Geometric morphometrics · Correlation networks · Variational modularity · Simulated annealing · Mammalian mandible

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Introduction

Complex phenotypes that function as integrated and cohesive units are heterogeneous systems with inherent attributes of diversity, individuality of components, and localized interactions among components (Wagner 1996; Levin 2003). A fundamental challenge in evolutionary biology is to identify such individual components as sets of statistically correlated phenotypic traits that are loosely coupled with other such sets of traits (Schlosser and Wagner 2004). Sets exhibiting such properties are defined as variational modules (Schlosser and Wagner 2004; Wagner et al. 2007). Variational modularity plays a fundamental role in the evolution of species because patterns of covariation at the phenotypic, macroscopic level may in turn affect the evolution of molecular networks (Wagner et al. 2007). In fact, the notion of modularity, in which the elements in a system are grouped into highly connected subsets (modules) that are more loosely connected to other such groups (Schlosser and Wagner 2004), has become a unifying principle to understand structure and function at

different levels of biological organization (Raff 1996; Winther 2001; Schlosser and Wagner 2004; Hintze and Adami 2008). At the molecular level, the architecture of developmental gene regulatory networks is modular, with components that execute specific functions, such as the specification of cell identity (Davidson and Levine 2008). Protein–protein interaction networks are also highly modular in structure (Wang and Zhang 2007). Recently, the statistical mechanical formalism of complex network theory has proven useful to discover and characterize modularity at the molecular level of organizational complexity (Ravasz et al. 2002; Guimerà and Amaral 2005; Newman 2006; Sales-Pardo et al. 2007; Kreimer et al. 2008; Ma'ayan 2009). Complex network theory, however, is conspicuously missing from the study of modularity at the phenotypic level, such as that of complex morphological structures. The network formalism represents a potentially powerful approach to the study of variational modularity because its tools do not rely on any preconceived notion of modularity (Newman 2006), leading to the detection of modules based solely on the patterns of interaction (Hintze and Adami 2008; Wang and Zhang 2007).

This article demonstrates for the first time the application of complex network theory and random matrix theory to the discovery and characterization of variational modularity. At this level of organizational complexity, the elements, or nodes, are quantitative traits and the connections, or edges, are measured as statistical correlations (Wagner et al. 2007). To achieve this goal, we used as a reference the mammalian mandible, a model system for the study of development and evolution of complex morphological structures (Atchley and Hall 1991; Hall 2003). The shape of the mammalian mandible at the macroscopic, morphological level reflects components whose individuality traces back to separate populations of cells called condensations. The formation and dynamics of condensations are, in turn, governed by different genes and gene cascades (Hall 2003). Measures of morphological variation in the mandible were derived from the methods of geometric morphometrics (Bookstein 1991; Adams et al. 2004). The geometric approach leads naturally to a network representation of quantitative morphological variation: samples of discrete points (landmarks) taken from phenotypic structures are the nodes and the edges are, as required, the statistics of correlation (Wagner et al. 2007). Here we integrate the tools of complex network theory and geometric morphometrics to answer the following questions: Can structural modularity be uncovered from complex morphological phenotypes? Is the modular structure biologically meaningful in the sense of corresponding to developmental, functional, and genetic units? Our results demonstrate that complex network theory can be at least as effective for the discovery and characterization of variational modularity as it has been for the understanding of the molecular level of organizational complexity.

Materials and Methods

Data

We analyzed a pooled sample of 51 male and female specimens of the echimyid rodent *Trinomys* sp. (*viz.*, *T. albispinus, T. bonafidae, T. dimidiatus, T. elegans, T. eliasi, T. iheringi, T. panema, T. paratus, T. setosus*). These species are closely related phylogenetically (Galewski et al. 2005) and are very similar in mandibular shape (Perez et al. 2009). The specimens included in this study were adults defined by the presence of the third molar with formed occlusal surfaces.

The mandible shape was captured as two-dimensional (2D) coordinates in a lateral digital image view (Fig. 1). Images of the mandible were obtained with an Olympus SP 350 digital camera. The *x*- and *y*-coordinates for 14 landmarks were recorded using the tpsDIG 2.10 software (Rohlf 2007). As in other studies, the landmarks defined here were chosen on the expectation that they approximate the skeletal regions derived from mesenchymal condensations involved in the development of the mammalian mandible (Atchely and Hall 1991). The landmarks are defined as follows: 1. The antero-dorsal border of the incisive alveolus (AlvA1); 2. The boundary between the molar alveolar and incisor alveolar on the dorsal curve of the mandible (AlvA2); 3. The anterior edge of the tooth-row (AlvP1); 4. The posterior edge of the tooth-row (AlvP2); 5. The tip of the coronoid process (Coron1); 6. The anterior-most point on the curve of the coronoid process (Coron2); 7. The anterior edge of the articular surface of the condyle (Cond1); 8. The tip of the condylar process (Cond2); 9. The posterior-most edge of the articular surface of condyle (Cond3); 10. The anterior-most point on the curve of the posterior boundary of the mandible (Ang1); 11. The tip of the angular process (Ang1); 12. The dorsal-most point on the ventral border of horizontal ramus (AlvP3); 13. The boundary between the incisor alveolar and mandible corpus on the ventral curve of the mandible (AlvP4). 14. The antero-ventral border of the incisive alveolus (AlvA3).

Superimposition and Statistical Analyses

The *x*- and *y*-coordinates for the 14 landmarks of the mandible were aligned by a Generalized Procrustes Analysis, whereby landmarks were optimally translated, scaled and rotated using a least squares criterion (Bookstein 1991; Adams et al. 2004). In addition, these coordinates were

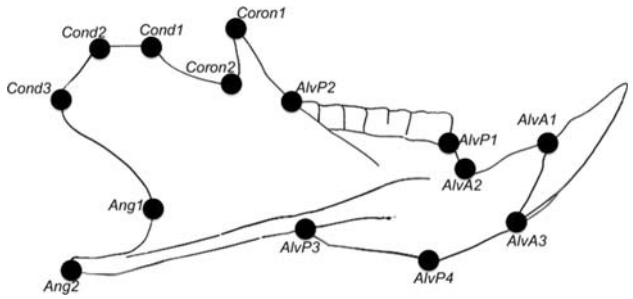

Fig. 1 The fourteen two-dimensional landmarks defined for the mandible

rotated around their common centroid to achieve the occlusal plane (see Klingenberg et al. 2003). These procedures generate an arbitrary, but functionally significant, orientation of the landmark coordinates, allowing compare with previous studies of rodent mandible (e.g. Klingenberg et al. 2003). The superimposition analyses were performed with the CoordGen6f software (Sheets 2003). Finally, we measured the associations among landmark coordinates using Pearson correlations and covariances. We used both measures because the correlations among landmark coordinates could have a major dependence on the orientation of the coordinate axes (Klingenberg and Zaklan 2000). In order to control for mean differences of the landmark coordinates among *Trinomys* species, correlations and covariances were estimated using residuals from the mean for each species (Marroig and Cheverud 2001; Porto et al. 2008). The residuals were calculated with a General Linear Model. The correlations and covariances obtained describe the linear relationship between two landmark coordinates. These statistics have been widely used in correlation networks analyses (Steinhauser et al. 2008).

Computation of the Optimal Correlation Coefficient Threshold

In the network representation of the mammalian mandible, the nodes are the anatomically defined landmarks points (Fig. 1) and the links among the landmarks were estimated as Pearson correlation coefficients. For construction of the correlation networks (Wagner et al. 2007; Steinhauser et al. 2008), the associations among landmark coordinates were combined into a square and symmetrical matrix with n landmark coordinates ($n \times n$ relationship matrix). The application of graph analyzing methods requires the conversion of the relationship matrix into a ($n/2 \times n/2$) binary adjacency matrix **A** (Steinhauser et al. 2008) of landmarks. Particularly, we then quantified the modularity of the mandible network using the algorithm of Guimerà and Amaral (2005), which is the most accurate method available in the literature to date (Danon et al. 2005). This algorithm nevertheless operates on binary matrices and, as a consequence, the matrix of Pearson correlation coefficients must be converted into a matrix of zeros and ones, the adjacency matrix (Albert and Barabási 2002). This is a crucial step in the correlation network analysis because the correlation threshold used for the discretization has to be chosen optimally (Steinhauser et al. 2008). The correlation threshold is the absolute value of correlation coefficient, r, below which a statistical interaction between nodes, that is, landmark points, is regarded as nonexistent and a value of zero is entered into the adjacency matrix (Steinhauser et al. 2008). In the opposite case, a value of 1 is assigned to the adjacency matrix. The binary adjacency matrix **A** has dimension $n/2$ because we used landmarks as the nodes and there are two associations (one for each landmark coordinates) that define the connection between two landmarks. Because a PROTEST analysis (with 10,000 permutations; Peres-Neto and Jackson 2001) showed a high and significant association between correlation and covariance matrices ($r = 0.6404$; $P = 0.0001$), we used the correlation matrix in the analyses.

Here we introduce a new two-step procedure to find the optimal correlation threshold. In the first step, we derived an interval for the optimal correlation threshold from the numerical behavior of the eigenvalues of adjacency matrices for different values of correlation thresholds (r), varying in their absolute values from $r = 0.05$ through 1.00, at 0.05 increments (Table 1). For a correlation threshold of $r = 0.05$, almost all eigenvalues of the corresponding adjacency matrix are identical. This happens because most elements in the correlation matrix exceed the correlation threshold of $r = 0.05$ and, consequently, most entries in the corresponding adjacency matrix will be ones (Table 1). As the correlation threshold increases from $r = 0.10$ to 0.20, the eigenvalues begin to vary in magnitude and as one gets to $r = 0.25$ all eigenvalues of the corresponding adjacency matrices are different (Table 1). A similar result is obtained for a correlation threshold of $r = 0.70$. Here all coefficients in the correlation matrix are smaller than the correlation threshold and, consequently, the corresponding adjacency matrix will have zeros in all entries (Table 1). Therefore, all eigenvalues of the adjacency matrix for this correlation threshold will also be zero. As the correlation threshold decreases from $r = 0.65$ to 0.50, the homogeneity of the eigenvalues of the corresponding adjacency matrices also decreases. Finally, at $r = 0.45$, most eigenvalues are different (Table 1). This numerical experiment demonstrates a change in the behavior of the correlation thresholds as inferred from variation in the magnitude of the eigenvalues of the corresponding adjacency matrices, and reveals an interval, from $r = 0.25$ to 0.45, which should contain the optimal correlation threshold. This interval was further narrowed using the criterion of statistical significance

Table 1 Eigenvalues of adjacency matrices for values of the threshold coefficient, *r*, ranging from $r = 0.05$ to 0.70, at 0.05 increments

$r = 0.05$	$r = 0.10$	$r = 0.15$	$r = 0.20$	$r = 0.25$	$r = 0.30$	$r = 0.35$	$r = 0.40$	$r = 0.45$	$r = 0.50$	$r = 0.55$	$r = 0.60$	$r = 0.65$	$r = 0.70$
−1.00	−1.87	−2.61	−3.09	−3.18	−3.20	−3.20	−2.69	−1.90	−1.85	−1.00	−1.00	−1.00	0.00
−1.00	−1.00	−1.82	−2.60	−2.52	−2.57	−2.57	−2.34	−1.82	−1.00	−1.00	0.00	0.00	0.00
−1.00	−1.00	−1.74	−2.00	−2.42	−2.30	−2.30	−1.83	−1.68	−1.00	−1.00	0.00	0.00	0.00
−1.00	−1.00	−1.00	−1.83	−2.04	−1.84	−1.84	−1.37	−1.00	−0.77	0.00	0.00	0.00	0.00
−1.00	−1.00	−1.00	−1.00	−1.59	−1.47	−1.47	−1.34	−1.00	0.00	0.00	0.00	0.00	0.00
−1.00	−1.00	−1.00	−1.00	−1.37	−1.00	−1.00	−0.69	−0.82	0.00	0.00	0.00	0.00	0.00
−1.00	−1.00	−1.00	−1.00	−0.64	−0.75	−0.75	−0.31	−0.54	0.00	0.00	0.00	0.00	0.00
−1.00	−1.00	−1.00	−1.00	−0.44	−0.61	−0.61	0.10	−0.35	0.00	0.00	0.00	0.00	0.00
−1.00	−1.00	−1.00	−0.56	−0.12	−0.21	−0.21	0.18	0.00	0.00	0.00	0.00	0.00	0.00
−1.00	−1.00	−1.00	0.23	0.30	0.44	0.44	0.52	1.00	0.00	0.00	0.00	0.00	0.00
−1.00	−1.00	−0.27	0.42	0.66	0.71	0.71	1.28	1.05	0.77	0.00	0.00	0.00	0.00
−1.00	−1.00	0.40	1.09	1.36	1.66	1.66	1.66	1.98	1.00	1.00	0.00	0.00	0.00
−1.00	0.00	0.83	1.25	2.11	2.48	2.48	2.49	2.21	1.00	1.00	0.00	0.00	0.00
13.00	12.87	12.20	11.09	9.88	8.65	8.65	4.33	2.87	1.85	1.00	1.00	1.00	0.00

(Steinhauser et al. 2008). At an alpha value of 0.05 and given our sample size, we arrive at $r = 0.35$ as the lowest significant correlation in our correlation matrix, resetting the correlation threshold interval between $r = 0.35$ and 0.45. In the second and final step, we used Guimerà and Amaral′s (2005) algorithm to find out which value of *r* inside this interval maximizes the statistic of modularity, *M* (see below). For the values of *r* used, 0.35, 0.40, and 0.45, the *M* statistic was calculated as 0.19, 0.36, 0.58, respectively. Therefore, the estimated optimal correlation threshold was $r = 0.45$ ($P = 0.005$) for a largest maximum *M* of 0.58.

Variational Modularity and Network Analyses

Network modularity was computed using a simulated annealing algorithm (Guimerà et al. 2004; Guimerà and Amaral 2005). Simulated annealing allowed us to estimate the modularity of any network generated using the optimal interval defined above without an a priori specification of the number of modules (Guimerà and Amaral 2005). The value of the modularity, *M* (Guimerà et al. 2004), for the matrix **A** was calculated as

$$M = \sum_{s=1}^{Nm} \left[\frac{ls}{L} - \left(\frac{ds}{2L} \right)^2 \right] \quad (1)$$

where *Nm* is the number of modules, *L* is the number of edges in the network, *ls* is the number of edges between nodes in module *s*, and *ds* is the sum of the degrees of the nodes in module *s*. Then, in order to test the significance of the modular structure of the original network, we calculate the modularity for 1,000 random graphs with the same degree (connectivity) distribution as the original network.

The deviation from the random structure of the adjacency matrix of the mandible network generated for the optimal correlation threshold was also investigated using methods from random matrix theory (Albert and Barabási 2002; de Aguiar and Bar-Yam 2005). The smoothed density of eigenvalues of the adjacency square matrix **A** is defined as

$$\rho_\varepsilon(\lambda) = \frac{1}{N} \sum_{i}^{S} \delta_\varepsilon(\lambda - \lambda_i) \quad (2)$$

where λ_i are the eigenvalues, and *N* is the total number of nodes in the network. δ_ε is a normalized Gaussian whose width ε controls the smoothness of density function (de Aguiar and Bar-Yam 2005). The smoothed density of eigenvalues allows a visual characterization of the network. In order to use it as a benchmark we also described the structure of the random (Wigner) matrix for our network (see additional details in Albert and Barabási 2002; de Aguiar and Bar-Yam 2005). A related approach based

on the eigenvalues distribution has been used previously to describe relationship matrices (Wagner 1984).

The network analyses were performed with Netcarto (Guimerà and Amaral 2005; Guimerà et al. 2004), Spectral series (de Aguiar and Bar-Yam 2005) and Pajek 1.23 software (Batagelj and Mrvar 2008). Procrustes analyses were performed using function protest in the vegan package for the R-system (Oksanen et al. 2008).

Results and Discussion

Application of the procedure described here yielded an estimated optimal correlation threshold of $r = 0.45$ ($P = 0.005$) for a largest maximum M of 0.58. The significance of the modular structure of the mandible network calculated with the optimal correlation threshold of $r = 0.45$ was then assessed by comparing it with a random network of the same size and distribution of links per node as the mandible network. The modularity of the mandible network ($M = 0.58$) is significantly larger than that of the random network, for which $M = 0.44$ (confidence interval; 0.40–0.48). The deviation from randomness of the mandible network was also assessed graphically by plotting the level spacing distribution of the eigenvalues of the adjacency matrix generated from the optimal correlation threshold for $r = 0.45$. This technique, originally from random matrix theory (Mehta 2004), has been used recently to investigate the topology and structure of molecular networks (de Aguiar and Bar-Yam 2005; Palla and Vattay 2006). For the randomized adjacency matrix of the mandible network generated for the optimal correlation threshold ($r = 0.45$), the level spacing distribution follows the well-known Wigner′s semicircle law (Fig. 2a). On the other hand, the level spacing distribution for the eigenvalues of the mandible network with an estimated M of 0.58 deviates remarkably from Wigner′s semicircle (Fig. 2b). Therefore, Guimerà and Amaral′s (2005) algorithm and random matrix theory are useful to study the modular structure of the mammalian mandible network.

The deviation from the random graph implies that the graph representing the mandible has an internal structure, whose modular nature was discovered using Guimera and co-workers' network method (Guimerà et al. 2004; Guimerà and Amaral 2005). We identified four modules in the mammalian mandible by Guimerà and Amaral′s (2005) algorithm for the optimal partition of the network in terms of landmarks points, as follows (Fig. 3): Landmarks AlvA1, AlvA3 and Cond3; landmarks AlvA2, AlvP1, AlvP2, AlvP3, and AlvP4; landmarks Coron1, Coron2, Cond1, and Ang1; and landmarks Cond2 and Ang2. These modules correspond with four morphogenetic units recognized for the mandible (Atchley and Hall 1991; Cheverud 2004), namely, the incisor and molar alveolar units and the anterior and posterior regions of the processes in the ascending ramus.

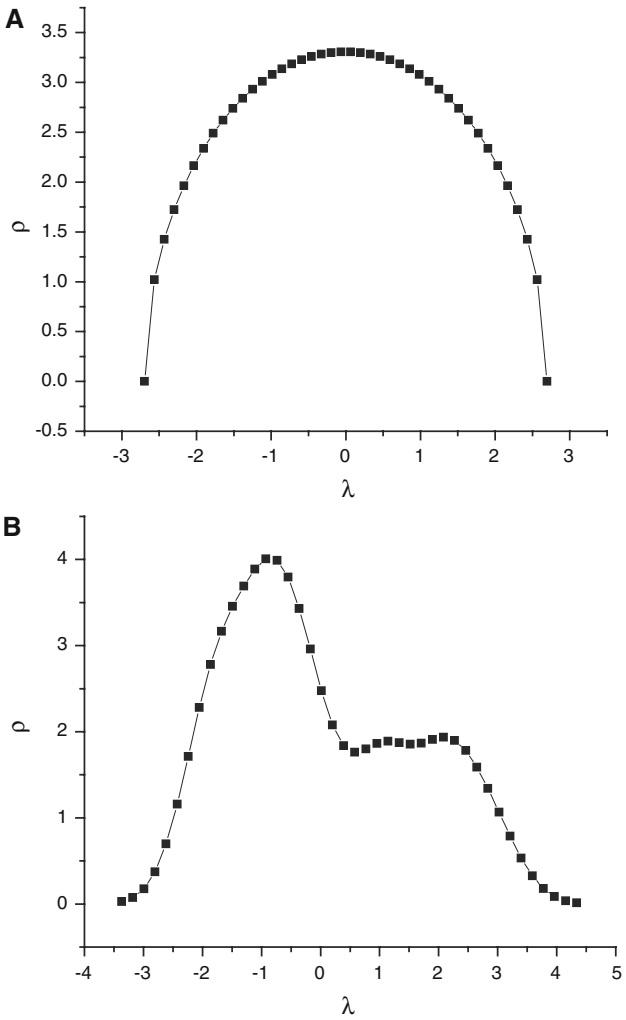

Fig. 2 **a** The smoothed density of eigenvalues for the randomized adjacency matrix. **b** The smoothed density of eigenvalues of the adjacency matrix of landmarks of the mandible

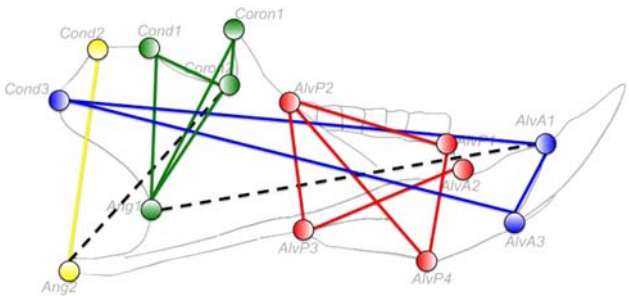

Fig. 3 The network for the adjacency matrix of landmarks of the mandible. The different colors represent the modules identified with the Guimerà et al. (2004) algorithm. The *dashed lines* represent connections between modules. (Color figure online)

The variational modules revealed in the mammalian mandible by complex network methods can be related to other levels of organizational complexity. At the genetic-molecular level, knockout experiments in mice demonstrate that some genes affect only particular mandible units. For example, the *msx-1* gene has effects on the teeth and associated alveolar bone, whereas the TGFß-2 gene is required for the growth of the three processes in the ascending ramus (Hall 2003). More generally, several studies of quantitative trait loci (QTL) by Cheverud and collaborators have shown that gene effects tend to be limited to specific developmental regions of the mandible (Cheverud et al. 1997; Ehrich et al. 2003; Cheverud 2004). In particular, Cheverud (2004) pointed out that 27% of studied QTL affected some individual mandible units (i.e. coronoid, condylar and angular process, incisor and molar alveolar), 44% have effects on the alveolar region or the posterior processes and 29% affect the whole dentary bone.

At the developmental, histogenic history level there is also evidence of modularity for the mandible. The ramal processes form by intramembranous ossification and then the cartilage is replaced by bone through endochondral ossification. The alveolar region, however, forms by intramembranous ossification, but from a condensation of cells that also differentiates into the fibroblasts of the periodontal ligament (Hall 2003). Finally, the mandible has regions that are functionally differentiated, thus displaying functional modularity (Cheverud 2004; Wagner et al. 2007). The posterior processes serve as a region for muscle attachment: the temporalis muscle is inserted into the de coronoid process, the lateral pterygoid is inserted into the condylar process, and the masseter and medial pterygoid muscles are inserted into the angular process and the corpus mandibular. In addition, the anterior portion of the mandible serves as the support for the incisor and molar teeth.

In summary, variational modularity could be influenced directly or indirectly by genetic, developmental and functional factors (Cheverud 2004; Hallgrimsson et al. 2007; Wagner et al. 2007). For example, maintenance of bone morphology depends on continuing interactions with tooth and muscle (Atchley and Hall 1991; Hall 2003; Cheverud 2004). The genetic and functional evidence of mouse mandible structure corresponds with our results, suggesting the importance of the muscle and teeth attachment region, as well as gene effects, to explain the variational modularity in the mammalian dentary bone. The modularity that we see in the mandible could result from the overlaying of these genetic, developmental and environmental factors and processes appearing during ontogeny, which determine the pattern of modularity among morphometric traits (Hallgrimsson et al. 2007; Mitteroecker and Bookstein 2009). Therefore, the mechanisms by which genetics, development and function contribute to the formation and maintenance of variational modularity could be relevant for understanding morphological variability and the evolutionary change. Nevertheless, it should be kept in mind that the multiple factors determining modularity can produce the same association structure (Hallgrimsson et al. 2007; Mitteroecker and Bookstein 2009).

The statistical mechanical formalism of complex network theory provides a powerful and robust insight into patterns of variational modularity and promises a new avenue to understand the origin and maintenance of phenotypic organization. This framework entails questions of detection and description of patterns of modularity and represents a critical initial step to test hypotheses about the factors and processes which determine modularity (Levin 1992). Moreover, the network approach may allow future studies to compare modularity across different levels of biological complexity.

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