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Morphological modularity and assessment of developmental processes within the vole dental row (*Microtus arvalis*, Arvicolinae, Rodentia)

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SUMMARY Knowledge of mammalian tooth formation is increasing, through numerous genetic and developmental studies. The prevalence of teeth in fossil remains has led to an intensive description of evolutionary patterns within and among lineages based on tooth morphology. The extent to which developmental processes have influenced tooth morphologies and therefore the role of these processes in these evolutionary patterns are nonetheless challenging. Recent methodological advances have been proposed allowing the inference of developmental processes from adult morphologies and the characterization of the degree of developmental integration/modularity of morphological traits by studying the patterns of variation within and among individuals. This study focuses on the geometric shape of the lower molars of the vole species *Microtus arvalis*. Our results suggest (i) quasi-independence of each molar at the developmental level (developmental modules), even slightly stronger for the third molar supporting some genetic and developmental hypotheses and (ii) more pervasive integration processes among molars at the morphological level.

INTRODUCTION

Mammalian tooth development has been investigated in depth since the end of the 19th century. First, biologists and paleontologists elaborated developmental hypotheses based on compared anatomy from embryo and adult phenotypic comparisons of characters to understand phylogenetic relationships (Bateson 1892; Butler 1939). They suggested that teeth were comparable with metameric structures (i.e., repetition of an identical unit of body organization; Le Moigne and Foucrier 2004), such as digits and vertebrae, evolving as parts of a system. In fact, both developmental and genetic control of tooth formation could act at the scale of tooth identity (i.e., molar, canine, and incisor: see Butler, 1939 morphogenetic fields, Osborn, 1977, 1978, cell clones; Weiss 1990). Modifications in development, such as genetic mutations, will primarily affect most or all teeth in a series (Butler 1939; Osborn 1978; Weiss 1990), and consequently teeth are not considered as separate evolutionary unit. Recent studies have improved our knowledge of tooth development without contradicting this previous hypothesis, which defines different morphogenetic processes as a reiterative signaling cascade (Jernvall and Thesleff 2000) of inhibitor and activator balance (Kavanagh et al. 2007) along the dental row. Different approaches have improved knowledge of the impact of development on the resulting tooth phenotype: variance-covariance matrices on common shrew tooth shape (Polly 2005), QTL on mouse molars (Workman et al. 2002), and finally morphodynamic models (see review in Salazar-Ciudad 2008). Despite diverging on the precocity and impact of the implicated developmental stages, all these approaches have underlined the role of development in resulting morphologies. Nevertheless, the extent to which adult phenotypic organization reflects underlying known tooth developmental mechanisms remains to be assessed. Phenotypic integration by developmental pathways can be estimated by the study of morphological modularity (e.g., bumblebee wings, Klingenberg et al. 2001; macaque and mouse skulls, Hallgrímsson et al. 2004; shrew mandibles, Young and Badyaev 2006; mammal skulls, Goswami 2007). Modularity is defined by the fact that organisms are divided into biological parts, which are hierarchically structured, and considered as semi-independent units both developmentally and structurally, with partial integration to ensure organismal coherence (Raff 1996; Wagner and Altenberg 1996; Winther 2001; Schlosser 2004).

A recent approach to the study of modularity and integration in developmental and evolutionary goals has been successfully used in several biological models (e.g., *Drosophila* wings, Klingenberg and Zaklan 2000; bumblebee wings, Klingenberg et al. 2001; mouse mandibles, Klingenberg et al.

2003). This approach makes inferences about the existence and the strength of developmental processes between modules from the covariation existing between morphological traits. The core idea is that if the traits are integrated – that is included in the same module – their morphological variation will be coordinated (Klingenberg 2008a). This method hypothesizes that such morphological covariation may result from two main developmental processes: parallel variation and direct interactions. Parallel variation arises between developmentally independent pathways, which are affected by an external genetic or environmental factor (Klingenberg 2005). Direct interactions take place between traits whose developmental pathways are linked (e.g., tissue partitioning or inductive signaling between pathways). Such interactions reveal intrinsic developmental constraints. Within a module, trait coherence will principally be ensured by direct interactions. Therefore, to detect modules from trait covariation, these two developmental processes need to be identified. To this end, trait covariation in fluctuating asymmetry (FA) levels is studied, in comparison with trait covariation in among-individual variation (IndVar). Each side of an organism shares the same genes and the same environment, apart from a few exceptions (see Klingenberg 2003). Thus, FA results from the randomness of development in each side of an organism (Palmer and Strobeck 1986; Auffray et al. 1999), and controls both environmental and genetic factors which are responsible for parallel variation of developmental pathways (Klingenberg 2003). Therefore, correlation of FA patterns can only arise if the developmental pathways responsible for these traits are affected by shared developmental processes (Klingenberg 2003). Thus, covariation of FA may be solely due to direct interactions between developmental pathways. However, trait covariation in IndVar can result both from direct interactions and from parallel variation of developmental pathways, so that developmental information is not distinguishable from IndVar (Klingenberg 2002, 2003, 2004). The study of trait covariation in FA can therefore infer the strength of direct developmental interactions among morphological traits and thus assess their degree of developmental modularity.

So far, most modularity and integration studies on mammals have dealt with skulls and mandibles (e.g., Cheverud 1982, 1995, 1996; Cheverud et al. 1997; Hallgrímsson et al. 2004; Bastir and Rosas 2005; Young and Badyaev 2006; Goswami 2007). Here, we focus more particularly on the lower molar row of voles. To date, several studies have suggested a genetic and developmental integration of groups of teeth by size (e.g., teeth of the carnassial region in Pengilly 1984; lower molar row in Workman et al. 2002 and in Kavanagh et al. 2007). The shape aspect is either examined in particular teeth (e.g., m1 in Polly 2005), or along the molar row (e.g., Workman et al. 2002). Thus, QTL analyses performed at the mouse molar row scale suggest genetic nonindependence of molar shapes (Workman et al. 2002). Therefore, by focusing on the vole molar row, our aim was to assess whether developmental integration also exists between molars with regard to shape.

Common vole teeth (*Microtus arvalis*) were chosen for this study, as tooth design in voles is suitable for the recognition of numerous morphological traits. Although mouse studies are more numerous, the vole is also a well-studied model, contributing greatly to knowledge of rodent cheek tooth development (e.g., Keränen et al. 1998; Jernvall et al. 2000; Salazar-Ciudad and Jernvall 2002; Matalova et al. 2005; Witter et al. 2005; Setkova et al. 2006). Several hypotheses have been presented to describe the modular organization of molars within the dentition (for an exhaustive review concerning tooth modular organization, see Stock 2001). The first hypothesis tested in this study assumes that within the molar row, all molars form an integrated unit. It results from the proposition that in mammals, the three tooth groups (i.e., incisor, canine, and molar) originate from three morphogenetic fields (Butler 1939, 1995). Morphogenetic fields probably constitute developmental modules (Gilbert et al. 1996; Klingenberg 2003, 2004). So the three molars developing from the same morphogenetic field could constitute a unique integrated unit. The resulting alternative hypothesis, also tested in this study, is that each molar could constitute a separate module (hypothesis evoked in Stock 2001, but not sufficiently supported by experimental data). A complementary hypothesis tested here is that the third lower molar is more genetically and developmentally independent than the other two, as observed in mice (Bader 1965) and humans (Silvestri and Singh 2003). Nevertheless, other possible causes are invoked for the independence of some teeth (see e.g., Pengilly 1984; Polly 1998; Meiri et al. 2005 about this debate). Consequently, with regard to these hypotheses, our aim was to specify whether or not the molar row shows integration between molar shapes. We also checked if the degree of integration (or modularity) in shape is lower (or higher) for the third lower molar in comparison with the other two. We applied geometric morphometric methods to decompose the sources of variation and to analyze the covariation among molars in order to characterize the integration patterns of molar shapes in the dental row, and to highlight the developmental processes that might be responsible for this structuring.

MATERIALS AND METHODS

Materials

The studied material is composed of 116 individuals from populations of the vole species *M. arvalis*, trapped in France [Vittel (Vosges), $N = 37$; Espezel (Aude), $N = 31$; and Parc de la Vanoise (Savoie), $N = 31$]. The material is stored in the "Centre de Biologie et de Gestion des Populations" Collections (Montpellier, France).

The three lower molars (m1, m2, and m3) were taken into account for all individuals.

Morphometrics

Landmark acquisition

Landmarks on the three lower molars of both sides were digitized using a Nikon MM-60 measuring microscope (Nikon-Japan, Tokyo, Japan) with a precision of 1 μm. Tooth orientation was normalized before landmark digitalization according to a plane surface (null *z*-coordinates), and the alignment of two landmarks: U and V for m1 (following Brunet-Lecomte 1988), W and X for m2 and Y and Z for m3 (this study, Fig. 1). Twenty-two landmarks (numbered from 1 to 22) were defined for m1, 12 for m2 (numbered from 23 to 34) and 13 for m3 (numbered from 35 to 47, Fig. 1). Except for landmarks 42 and 43 (m3), they all correspond to the maximal curvature points of the salient angles (called "triangles"), loop tips and re-entrant angles of the tooth. They therefore correspond to landmarks of type II following the nomenclature of Bookstein (1991). Left and right teeth were re-positioned and digitized twice for each individual in order to assess measurement error (ME) caused by positioning and digitizing. The analyses presented here were processed with Matlab® programming and MorphoJ software (Klingenberg 2008b).

Geometric morphometrics

In geometric morphometrics, shape is defined as all the geometric information about an object invariant to size, location, and orientation (Dryden and Mardia 1998). Shape variation was extracted from landmark configuration using generalized least-square Procrustes superimposition. This approach normalizes all configurations to the same centroid size, superimposes them on the same centroid, and rotates them according to a least-square criterion. Before these three traditional steps, in the case of FA analysis, the Procrustes method proceeds by first reflecting one side to its mirror image to align corresponding landmarks on both sides (Klingenberg and McIntyre 1998). Procrustes-aligned configurations are projected on to a linear tangent space at the mean shape (Dryden and Mardia 1998). The tangent coordinates can then be processed using all sets of multivariate linear analyses.

Procrustes fitting was performed separately for each tooth. In fact, the relative freedom of teeth within the alveolus, implying for example differences in the occlusal plane among teeth, could include nonbiological information about tooth relative position. Therefore, the shape quantified here refers to aspects of shape of the molars themselves: that is all the geometric information about the cusps constituting the molars after removing the size, position, and orientation of the teeth. In opposition, the shape quantification obtained from a single Procrustes superimposition of the three molars would also incorporate shape features related to the molar row: in that case shape would also contain the relative sizes and orientations of the molars within the molar row.

Preliminary analyses

From tangent coordinates of individual tooth configurations, Mahalanobis distances were computed for each side and each replicate to detect outliers. A sequential procedure was used where all landmark configurations (three molars × two sides × two replicates) of the strongest outlier (i.e., with the strongest distance value) were removed before a new Procrustes fitting and the re-computation of the Mahalanobis distances. All these outlier individuals were definitely removed before the remaining analyses.

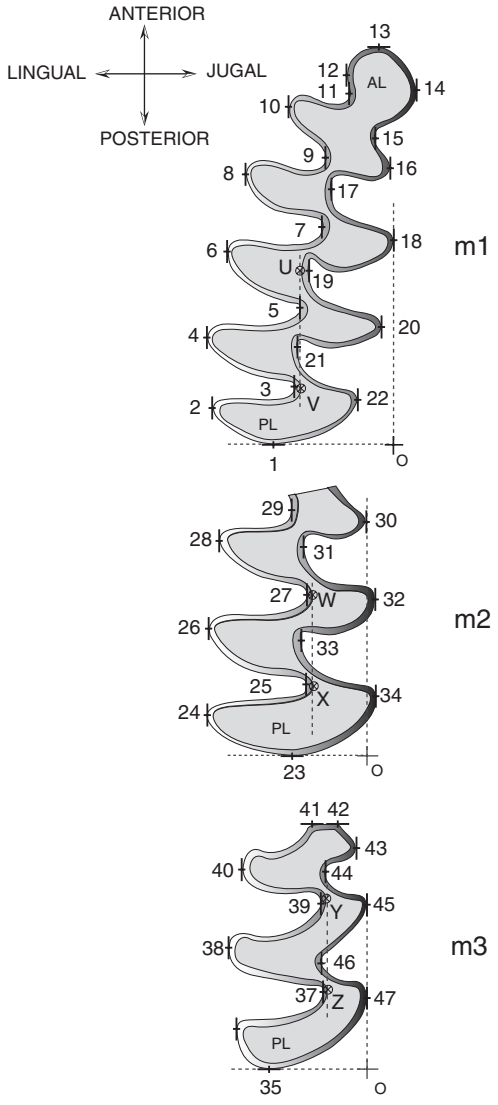

Fig. 1. Description of landmarks defined in this study. The occlusal areas of the three molars are represented. Orientation of each tooth follows an axis defined by two landmarks (crossed circles from U to Z) on the internal enamel outline. Landmarks 1–47 on the external enamel outline are those used for morphometrics.

Individual values of signed asymmetry were computed as the left–right differences between Procrustes coordinates corrected for mean asymmetry, to obtain a measure of FA (Palmer and Strobeck 1986). Data were also corrected for the main effect of population size. A Procrustes analysis of variance (ANOVA) was performed (Klingenberg and McIntyre 1998; Klingenberg et al. 2002): it corresponds to a geometric morphometric extension of the two-way mixed model ANOVA of Palmer and Strobeck (1986) for the study

of asymmetry. This approach allows the assessment of the significance of shape FA compared with ME. Potential allometric relationships in FA were tested and corrected by multivariate regression of the signed asymmetries of shape on centroid size (Monteiro 1999; Klingenberg et al. 2003).

Modularity analyses

Tested hypotheses

Several comparisons were tested with regard to two possible situations, that is, whether the molar row constitutes a single integrated unit, or each molar is an independent module. First, we tested the null hypothesis that the three teeth were three independent modules (m1/m2/m3). Then we focused on the pairwise relationships among molars: m1/m2, m1/m3, and m2/m3. Finally, we examined possible stronger independence of the first molar against the last two (m1/m2m3) and the third molar against the first two (m1m2/m3).

Quantifying covariation

Here, we used a methodology developed recently (Klingenberg and Zaklan 2000; Klingenberg et al. 2001, 2003), which uses IndVar and FA comparisons to assess developmental process responsible for modularity. Further improvement of this method uses the R_v coefficient (Escoufier 1973; Klingenberg 2007). This coefficient is based on the joint covariance matrix (noted $\mathbf{S}$) of two sets of tangent coordinates ($\mathbf{E}$ and $\mathbf{F}$ with, respectively, k_1 and k_2 landmarks in m dimensions):

$$\mathbf{S} = \begin{pmatrix} \mathbf{S}_1 & \mathbf{S}_{21} \\ \mathbf{S}_{12} & \mathbf{S}_2 \end{pmatrix} \quad (1)$$

with $\mathbf{S}_1$ and $\mathbf{S}_2$ the two covariance matrices of the sets, and $\mathbf{S}_{12}$ the cross-covariance matrix between the two sets (with $\mathbf{S}_{12} = \mathbf{S}'_{21}$). R_v corresponds to:

$$R_v = \frac{\text{trace}(\mathbf{S}_{12} \times \mathbf{S}_{21})}{\sqrt{\text{trace}(\mathbf{S}_1^2) \times \text{trace}(\mathbf{S}_2^2)}}, \quad (2)$$

where the numerator is

$$\text{trace}(\mathbf{S}_{12} \times \mathbf{S}_{21}) = \sum_{j=1}^{m \times k_1} \sum_{h=1}^{m \times k_2} \text{cov}(\mathbf{e}_j, \mathbf{f}_h)^2 = Xcov, \quad (3)$$

where $\text{cov}(\mathbf{e}_j, \mathbf{f}_h)$ is the covariance between the jth variable of set 1 and the hth variable of set 2 (the element of the jth row and hth column of the cross-covariance matrix). Therefore, the numerator of R_v (named $Xcov$) is equal to the total squared cross-covariance between the two sets, which is used as a summary statistic in partial least squares analysis (PLS; Rohlf and Corti 2000) to quantify the total amount of cross-covariance.

The elements of the R_v denominator are

$$\text{trace}\left(\mathbf{S}_1^2\right) = \sum_{j=1}^{m \times k_1} \text{var}(\mathbf{e}_j)^2 + \sum_{h=1}^{m \times k_1} \sum_{g=1 \wedge g \neq h}^{m \times k_1} \text{cov}(\mathbf{e}_h, \mathbf{e}_g)^2 \quad (4)$$

which correspond to the sum of the squared variances and covariances within each set. The equivalent is obtained for $\text{trace}(\mathbf{S}_2^2)$ by changing the appropriate number of landmarks and variables. Therefore, the R_v denominator is the squared root of the product of the sum of squared variances and covariances of each set.

Consequently, this R_v coefficient measures the association between two subsets of landmarks, by quantifying inter-subset covariation, normalized by intra-subset variation and covariation. Taking all variances as equal to σ^2 and all correlations between variables as equal to 1, then the total cross-covariance and the R_v denominator are equal to $(m \times k_1) \times (m \times k_2) \times \sigma^2$ which corresponds to the maximal possible value of total cross-covariance given by Rohlf and Corti (2000) in the PLS analysis context (with $\sigma^2 = 1$). Therefore, R_v coefficient is a generalization to unequal variances of the second summary statistic used in PLS analysis: the total amount of cross-covariance between sets, normalized by its maximal possible value. It will reach its maximal value of 1, when all the correlations between variables equal 1 and more generally when $\mathbf{F}$ is an orthogonal transformation of $\mathbf{E}$ because R_v is invariant to such orthogonal transformations affecting $\mathbf{E}$ and $\mathbf{F}$ (i.e., rotation, symmetry, and scaling: Escoufier 1973; Klingenberg 2007). Conversely, R_v will be equal to 0 when all cross-covariances are null.

For cases where the covariation is quantified over the three molars simultaneously, the R_v coefficient was computed for each pairwise comparison and then averaged (Klingenberg 2007).

Hypothesis testing

Using the R_v coefficient, we quantified the covariation among teeth on averages of the left and right teeth of individuals (i.e., IndVar) as well as on the signed asymmetries (i.e., FA, computed as differences between the left and right teeth corrected for mean asymmetry). Developmental modules are characterized by low covariation between them due to direct developmental interactions (Klingenberg 2005). Therefore, a low R_v value on signed asymmetries will be expected between developmental modules. Because covariation of IndVar arises from more factors (e.g., size, genetics, environment), higher covariation is expected than for FA. Complete modularity of teeth (i.e., all teeth independent) will be attained if covariation among teeth is not significant. However, modularity is a matter of degree more than an all-or-nothing matter (Klingenberg et al. 2003).

We assessed this null hypothesis of complete modularity ($R_v = 0$) using a permutation approach (initially used with R_t coefficient, Klingenberg et al. 2003, and extended here to R_v). We randomly exchanged teeth among individuals, and recomputed a new random R_v. This procedure was repeated 10,000 times and yielded a distribution of R_v under the null hypothesis of null covariation. A permutation P-value was then obtained as the number of permuted R_v greater or equal to the observed values divided by the number of permutations processed minus 1.

PLS analysis

The R_v coefficient quantifies the overall covariation between teeth, and thus complements PLS. PLS uses the singular value decomposition of the matrix $\mathbf{S}_{12}$ to extract pairs of axes that are uncorrelated with other PLS pairs and accounted for a decreasing amount of squared cross-covariance (Sampson et al. 1989; Klingenberg and Zaklan 2000; Rohlf and Corti 2000). Thus, PLS analysis allows independent features of shape covariation to be quantified and visualized.

As explained before, in the PLS framework, the total amount of cross-covariance between parts is usually computed (Rohlf and

Corti 2000). It corresponds to the sum of squared singular values, and therefore to the sum of the squared cross-covariances. This is *Xcov* (the numerator of the R_v coefficient, see Equation 3). Rohlf and Corti also proposed a scaling of this total cross-covariance by its maximum possible value, which in the case of the correlation matrix, is the product of the number of variables in the two sets. As stated above, the R_v coefficient is the generalization to the case of unequal variances of the amount of cross-covariance, scaled by the amount of intrablock variance and covariance.

Correlation between the individual scores on the two axes of each PLS pair can also be computed. This correlation refers to the strength of integration of shape features represented by each axis (Bookstein et al. 2003; Bastir and Rosas 2005).

We assessed the significance of between tooth covariation accounted for each PLS pair of axes using the same permutation procedure as for R_V, also repeated 10,000 times. We recomputed the singular values of the cross-covariance matrix between the teeth. This procedure simulated a null hypothesis of null covariation as with the R_v coefficient, but allowing (i) the amount of cross-covariance for each feature of covariation (i.e., each PLS pairs), and (ii) the significance of the correlation between the scores on each PLS pairs (simulating $r = 0$) to be tested. *P*-values were obtained by counting the number of times where the random singular values, or the random correlations, were higher than those observed at the same rank of PLS pairs.

RESULTS

Preliminary analyses

Seventeen individual outliers were detected, based on Mahalanobis distances, and excluded for following analyses. The 99 remaining individuals were then analyzed. Procrustes ANOVA showed that FA was highly significant relative to ME for each molar (see Table 1), and could therefore be used for analyses of modularity.

Table 1. Procrustes ANOVA of the shape variation

	Effect	Sums of squares	Mean squares $\times 10^3$	Degrees of freedom	*F*	*P*
m1	Individuals	0.7702	0.1965	3920	6.57	<0.0001
	Sides	0.0101	0.2531	40	8.46	<0.0001
	Ind. sides	0.1173	0.0299	3920	4.56	<0.0001
	Error 1	0.052	0.0066	7920		
m2	Individuals	1.0682	0.545	1960	6.1	<0.0001
	Sides	0.0142	0.7111	20	7.95	<0.0001
	Ind. sides	0.1752	0.0894	1960	4.09	<0.0001
	Error 1	0.0866	0.0219	3960		
m3	Individuals	1.2831	0.5951	2156	5.34	<0.0001
	Sides	0.0318	1.4444	22	12.97	<0.0001
	Ind. sides	0.2401	0.1114	2156	4.2	<0.0001
	Error 1	0.1154	0.0265	4356		

Sums of squares and mean squares are in squared Procrustes distance units following (Klingenberg and McIntyre 1998).

Modularity analyses

Quantification of covariation and significance levels (Table 1)

For the IndVar, the tested m1/m2/m3 separation is characterized by low, but highly significant, intertooth covariation ($Rv = 0.19$, $P < 0.0001$), whereas for FA, the R_v value of 0.11 is nonsignificant ($P = 0.39$). Thus, the low and nonsignificant R_v for FA suggests that the strength of direct developmental interactions among molars is weak and that the three molars could constitute three developmentally independent units of the molar row.

As the R_v is the result of an average between the three molars (see Materials and methods), this result could mask the presence of nonindependent units. As a consequence, paired comparisons must be tested in order to investigate more precisely the covariation relationship between pairs of molars. For the three pairwise comparisons tested (m1/m2, m1/m3, and m2/m3), the R_v values for FA are of similar order, increasing slightly from the m2/m3 comparison with the m1/m2 comparison (see Table 2).

There is considerable difference between the R_v for IndVar and the R_v for FA in the m2/m3 comparison. The R_v value for IndVar is more than twice that for FA. Significance levels of R_v for FA are contrasted: $P = 0.08$ for m1/m2, $P = 0.60$ for m2/m3, and $P = 0.74$ for m1/m3. As a result, developmental independence of each molar is never rejected, but a lower *P*-value could indicate a higher dependence between m1 and m2, compared with m3.

To reinforce this result, independence of m1, and then m3, was investigated further by looking at their respective m2 combinations. Combination m1m2/m3 shows a lower level of covariation for FA ($R_v = 0.13$, $P = 0.70$) than the m1/m2m3 ($R_v = 0.16$, $P = 0.32$). These elements confirm the higher relative independence of m3, compared with the m1m2 association.

Table 2. R_v coefficient values for the different tested comparisons

	IndVar		FA	
	R_v	*P*	R_v	*P*
m1/m2/m3	0.19	<0.0001	0.11	0.39
m1m2/m3	0.26	<0.0001	0.13	0.70
m1/m2m3	0.23	<0.0001	0.16	0.32
m1/m2	0.19	<0.0001	0.12	0.08
m1/m3	0.17	<0.0001	0.12	0.74
m2/m3	0.23	<0.0001	0.09	0.60

IndVar, individual variation; FA, fluctuating asymmetry.

PLS analysis

Cross-covariation analyses between adjacent pairs of molars (m1/m2 and m2/m3) are represented by two sets of PLS analyses (Fig. 2, A and B).

For both IndVar and FA, the total amount of squared cross-covariance (*Xcov*) is very low between both m1 and m2, and m2 and m3, but *Xcov* is significant only for IndVar ($P<0.0001$). Although *Xcov* was nonsignificant for FA, the PLS for FA was computed for the purpose of comparison with the among-individual PLS. For IndVar, the amount of cross-covariance accounted for the PLS pairs was significant for the first nine (m1/m2) and seven (m2/m3) PLS pairs. However, intertooth correlation was significant only for the PLS1 of the m2/m3 comparison in IndVar (Table 3).

Although the features of intertooth correlation are rarely significant, we have represented the first two PLS pairs to compare this intertooth correlation between FA and IndVar (Fig. 2). In the case of the m1/m2 comparison, the first two axis pairs explained just over 62% of intertooth covariation for IndVar, and just under 50% for FA. For IndVar, the two PLS axis pairs mainly characterize associated bends (due to

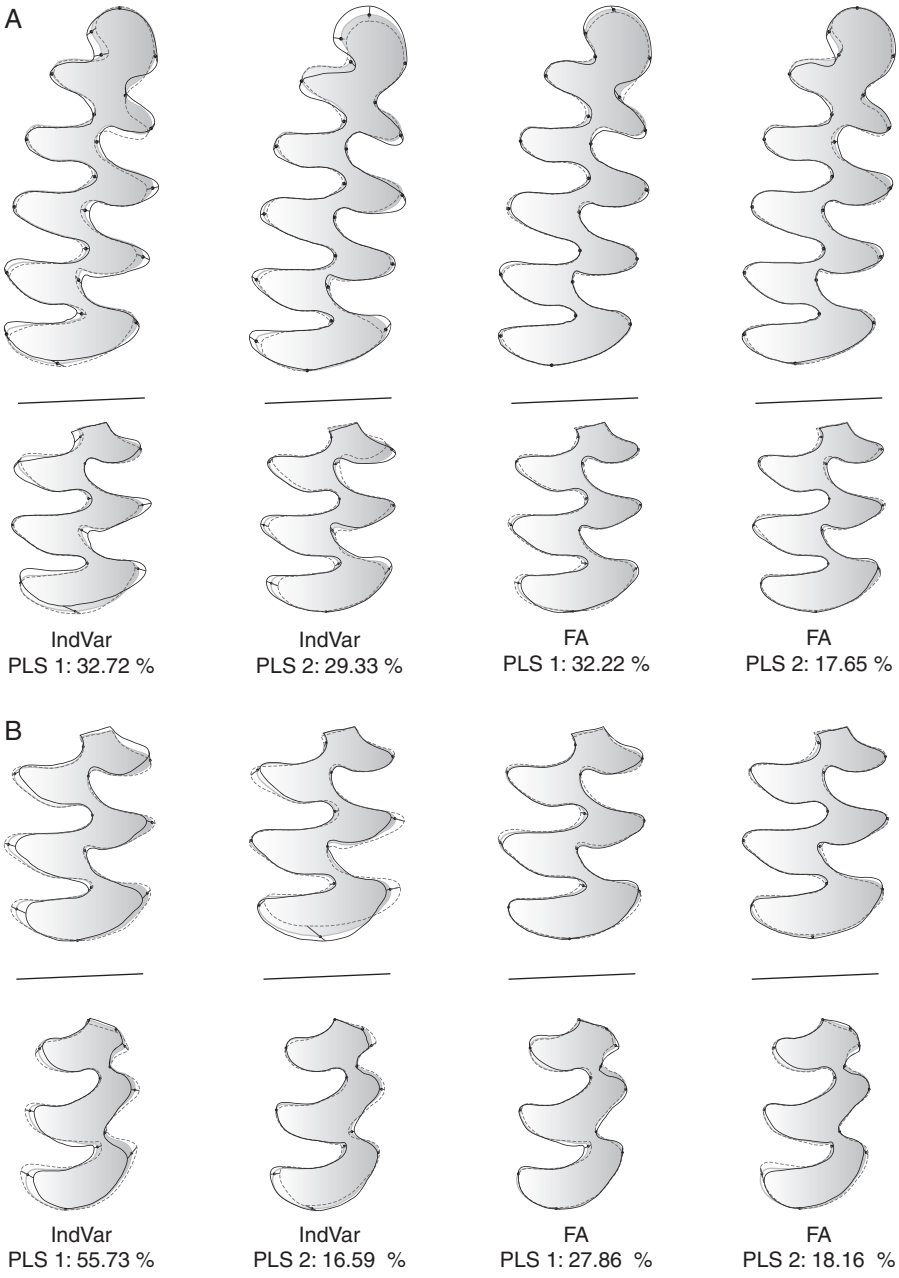

Fig. 2. Patterns of shape intertooth covariation displayed by the first PLS axis pairs for fluctuating asymmetry (FA) and individual variation (IndVar) between m1/m2 (A), and m2/m3 (B). Circles constitute the consensus configuration, whose possible outline is represented by the bold gray line. The maximum attained deviations for each circle are shown as black segments and outline (negative side), and dashed gray segments and outline (positive side) for the axis pair under consideration. Percentages of explained covariation for each PLS pair between teeth are also reported.

Table 3. Details of PLS analysis results

	Singular value $\times 10^3$	P	Percentage of intertooth covariation	Correlation	P
m1/m2					
IndVar					
PLS1	0.19	0.02	32.72%	0.54	0.29
PLS2	0.18	<0.0001	29.33%	0.55	0.82
Xcov $\times 10^7$	1.16	<0.0001			
FA					
PLS1	0.09	0.31	32.22%	0.45	0.96
PLS2	0.07	0.20	17.65%	0.45	0.83
Xcov $\times 10^7$	0.25	0.08			
m2/m3					
IndVar					
PLS1	0.35	<0.0001	55.73%	0.63	0.02
PLS2	0.19	<0.01	16.59%	0.51	0.11
Xcov $\times 10^7$	2.29	<0.0001			
FA					
PLS1	0.11	0.81	27.86%	0.38	0.77
PLS2	0.09	0.57	18.16%	0.44	0.33
Xcov $\times 10^7$	0.43	0.60			

IndVar, individual variation; FA, fluctuating asymmetry; PLS, partial least squares.

triangle lengthenings and inclinations) often localized within entire teeth, or sometimes within specific parts of m1 (jugal and posterior parts). In m1, the anterior loop shows specific patterns of dilatation, closure or shear (Fig. 2A). Patterns of covariation for FA are somewhat different, as they are more often localized in specific parts (jugal or anterior parts of m1, jugal, lingual, or anterior parts of m2). These patterns are generally bends and anterior loop deformations (closure, shear) (Fig. 2A).

In the case of the m2/m3 comparison, the first two axis pairs encompass more than 72% of intermolar covariation for IndVar, and almost 46% for FA. The first two PLS axis pairs for IndVar display triangle lengthenings and inclinations, and lingual/jugal asymmetries between m2 and m3. They are localized either in the entire molars or in specific parts (posterior loops and anterior parts, Fig. 2B). For FA, the patterns depicted by the PLS axes differ, as they concern more localized parts of teeth (posterior loops, anterior parts, or isolated triangles) and display loop or triangle asymmetries and inclinations (Fig. 2B).

DISCUSSION

To assess the modular organization of the *M. arvalis* molar row, two hypotheses can be proposed: either the three teeth constitute semi-independent modules (Stock 2001), or the three molars are a single integrated block with many developmental interactions (resulting from their common developmental origin within their shared morphogenetic field, Butler 1939). Our results show that, for shape covariation, the R_v coefficient is always comprised between 0.09 and 0.26 for both FA and IndVar. Comparing these values with the theoretical range of R_v (0–1), they could indicate a weak shape covariation between teeth. As a comparison, the only study using the R_v coefficient for modularity was conducted on *Drosophila* wings. It suggests that anterior and posterior compartments are not independent modules, with an R_v value $= 0.41$ (Klingenberg 2007). However, a direct comparison between the two studies remains difficult, because of the different organisms and traits studied (Klingenberg 2007).

The nonsignificant amount for covariation of FA and, therefore, the nonrejection of null covariation among tooth shapes, strengthens the idea that molars could constitute three independent developmental modules, whatever the scale of observation (two or three teeth). Nevertheless, this could also be due to a weak statistical power for FA. In contrast, for IndVar, which takes into account both direct interactions and parallel variation, covariation is low but always significant. Furthermore, R_v values of IndVar analyses were consistently higher than those obtained for R_v values of FA, suggesting the presence of additional components of shape covariation among the three teeth.

Our results on FA suggest that molars are not integrated within the molar row by direct interactions between their developmental processes, but that more pervasive factors of morphological integration act at the level of IndVar. Therefore, the vole molar row could be a unique morphological module including three developmental modules (Fig. 3). This pattern of shape modularity diverges from the hypothesis of integrated cheek teeth (Butler 1939, 1995) if we only focus on developmental integration, but is congruent to molar row morphological integration. What processes cause such a divergent pattern of integration?

In FA, shape covariation is primarily localized within each molar. Trait covariation in FA can result from direct interactions, for example inductive signals between pathways or precursor partitioning (Klingenberg 2003, 2004); such processes can act within each molar. Possible candidates for precursor partitioning are cells of the primary enamel knot controlling molar formation which divide into secondary enamel knots controlling particular cusp formation (Jernvall et al. 2000). From experiments and models on developmental mechanisms in rodent molars, Jernvall and Thesleff (2000), and Kavanagh et al. (2007) demonstrated that cheek tooth development corresponds to a reiterative signaling cascade (m2 inhibited by m1, and m3 by m1 and m2, but voles seem to be a particular case: Polly 2007, Renvoisé et al. 2009), induced by a balance between activators and inhibitors. This balance influences molar timing and size. On our data (results

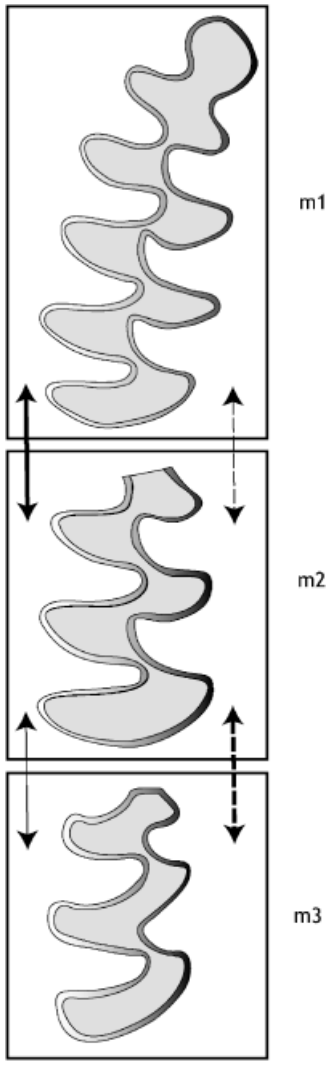

Fig. 3. Summary of shape modular organization and developmental processes involved in the vole dental row. Modules are represented by black rectangles. Direct interactions are shown as continuous black arrows, and parallel variation as dashed black arrows. Differential arrow thickness qualitatively indicates relative importance of direct interactions and parallel variation between teeth.

not shown), such strong correlation of molar sizes is found (on average $r = 0.79$). Thus, if an inductive signal is implied within each molar, a similar inhibition cascade could act at the molar scale and generate shape covariation. Also, the inhibitory cascade evoked by Kavanagh et al. (2007) might generate direct interactions via inductive signaling pathways between molar traits. As this signaling cascade affects the relative sizes of molars (Kavanagh et al. 2007), it could be a more pervasive factor for integration at the level of the molar row shape. This could be responsible for the small and non-significant covariation found in FA.

In IndVar, an additional part of shape covariation could act between molars. Parallel variation and direct interactions are thought to explain trait covariation in IndVar. Parallel variation is caused by genetic or environmental factors (Klingenberg 2003, 2004). Such genetic factors for morphological integration could be a common genetic basis of tooth shape as shown by QTL studies on mice (Workman et al. 2002). Detected QTLs did not show any regionalization of their effects for different molars and were highly correlated (Workman et al. 2002). Polymorphisms of such genes implied in tooth shape developmental pathways, but expressed downstream to some inductive signaling among pathways (e.g., inhibitory cascade, Kavanagh et al. 2007), or to some precursor partitioning, could affect different teeth independently but in a similar way and therefore could produce among-individual shape covariation. Similarly, environmental factors and their complex interactions with genes could also lead to among-individual covariation. Such complex responsiveness of molar shape to environmental context for different genetic backgrounds has been shown for example in the case of prenatal exposure to dioxin (mice: Keller et al. 2007a, 2008). Moreover, in variational environmental contexts, dental wear could also induce such covariation between teeth, as it impacts on the whole molar row.

As stated above, our results partly echo the signaling cascade affecting the relative sizes of molars (Kavanagh et al. 2007). This cascade could likely be a more pervasive factor of integration at the level of the molar row shape (i.e., all the geometric information about the three molars after one single Procrustes superimposition removing the size, location and orientation of the molar row). Therefore, an analysis performed by the dental row scale would probably give a different result for FA, with an expected higher developmental integration of the three molars. Such potential discrepancies come from the definition of the molar row shape versus the molar shapes. For the row shape, the relative sizes and orientations of molars will be conserved but not for molar shapes. We must emphasize that these two shape definitions are complementary. Both shape quantifications have their own biological meaning. An approach using both definitions together could highlight the anatomical features where developmental integration occurs. This approach might also provide additional insights into the nature and in the strength of tooth integration in the molar row.

Results of this study suggest a modular developmental organization of shape with three individualized molars. Nevertheless, modularity is a hierarchical concept (Raff 1996; Wagner and Altenberg 1996; Bolker 2000; Eble 2005; Klingenberg 2005; Rasskin-Gutman 2005; Thomas 2005) and the hypothesis of integrated cheek teeth (Butler 1939) must certainly not be rejected at a higher level of observation. At the scale of the mandible, the molar family could constitute an integrated unit, like the incisor family. This could reflect the idea that the hierarchical organization of modularity mirrors successive stages in development (Raff 1996;

Klingenberg 2004): the highest levels correspond to the first stages of development; as a corollary, the lower levels reveal later stages. In the case of teeth, development is characterized by the formation of a molar morphogenetic field which then divides into three molar zones (Butler 1939; Jernvall and Thesleff 2000). The modular organization observed in the three molars could express this developmental stage. To pursue this idea, in the later stages of development, the individualization of cusps would therefore represent independent modules at the finest scale.

For the particular case of m3, both the three pairwise comparisons and the "two against one" comparisons detail the degree of modularity within the molar row. Our results suggest that covariation of shape in the m1/m2 comparison is higher and more significant than in m2/m3, or in m1/m3. This is reinforced by the lower degree of covariation found between m3 and the m1m2 group in FA. Thus, it can be suggested that the m1 and m2 molars are better integrated in the shape aspect than m3, which seems to be more developmentally independent (Fig. 3).

Another aspect highlighted by Kavanagh et al.'s model (Kavanagh et al. 2007) is the atypical behavior of the m3 molar during development. This tooth erupts a few days later than m1 and m2 (e.g., Kavanagh et al. 2007 for mice). The third molar may be of less than average size (mice, Bader 1965; humans, Silvestri and Singh 2003). It may even be absent in some mouse laboratory strains through mutations (e.g., Nomura et al. 2003) or susceptibility to environmental toxicants (Keller et al. 2007b), or absent at higher taxonomic level in some mammal families (e.g., Hillson 2005). Because of the peculiar development of the third molar, we would expect it to display less covariation in shape with m1 and m2, and as an outcome, to display a higher degree of modularity. Our results seem to confirm this higher degree of independence of m3.

Using geometric morphometric techniques, we were able to highlight interactions between developing parts like teeth through covariation between different phenotypic traits. Developmental processes following Klingenberg's dichotomy (direct interactions vs. parallel variation) can partly echo the activator–inhibitor balance involved in tooth development. Thus, the ability to infer developmental processes from the decomposition of morphological covariation opens fundamental evolutionary perspectives leading to studies of development among natural populations. Nevertheless, this approach is restricted to situations where FA can be estimated (i.e., situations where left and right sides are present, identifiable and constitute a sufficiently large dataset).

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