Original Paper

Title: Patterns of morphological variation in enamel-dentin junction and outer-enamel surface of human molars

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Abstract

Tooth crown patterning is governed by the growth and folding of the inner enamel epithelium (IEE) and the following enamel deposition forms outer enamel surface (OES). We hypothesized that overall dental crown shape and covariation structure is determined by processes that configurate shape at enamel-dentin junction (EDJ), the developmental vestige of IEE, and tested this hypothesis by comparing patterns of morphological variation between EDJ and OES in human maxillary permanent first molar (UM1) and second deciduous molar (um2). Using geometric morphometric methods, we described morphological variation and covariation between EDJ and OES, and evaluated the strength of two components of phenotypic variability: canalization and morphological integration, in addition to the relevant evolutionary flexibility, i.e., the ability to respond to selective pressure. The strength of covariation between EDJ and OES was greater in um2 than UM1, and the way that multiple traits covary between EDJ and OES were different between these teeth. The variability analyses showed that EDJ had less shape variation and a higher level of morphological integration than OES, which indicated that canalization and morphological integration acted as developmental constraints. These tendencies were greater in UM1 than um2. On the other hand, EDJ and OES had the comparable level of evolvability in these teeth. Amelogenesis could play a significant role in tooth shape and covariation structure, and its influence was not constant among teeth, which may be responsible for the differences in the rate and/or period of enamel formation.

Key Words: developmental constraints, geometric morphometrics, morphological variability, evolvability, odontogenesis

Introduction

Dental morphological characteristics such as cusps, accessory cusps, and ridges on the occlusal surface have been used extensively in the studies of hominoid evolution and phylogenetic relationships (Miller, 1918; Simons and Pilbeam, 1972; Dean, 2000; Pilbrow, 2006; Matsumura et al., 2011). Tooth crown morphology is determined through two developmental processes (Avishai et al., 2004; Skinner and Gunz, 2010; Smith et al., 2011). The first process is the growth and folding of the inner enamel epithelium (IEE) during the bell stage. This morphogenesis (= tooth crown patterning) is governed by interactions between the IEE and underlying mesenchymal tissues. The final configuration of the IEE is preserved as the enamel-dentin junction (EDJ). The second process is biomineralization by the enamel-forming ameloblasts and dentin-forming odontoblasts. Ameloblatsts are derived from the IEE cells and odontoblasts from the dental papilla cells. Enamel formation starts at the cusp tips, and proceeds apically to complete the outer-enamel surface (OES).

Recent micro-CT dental analyses have revealed that crown morphological traits of the completed EDJ are modified or masked through the process of enamel deposition (Skinner et al., 2009, 2010; Ortiz et al., 2012), and that the extent of modification varies depending, in part, if not totally, on the enamel thickness (Ortiz et al., 2012). This raises a concern about whether or not shared derived features and homoplastic features of similarity at the OES can be properly discriminated (Hunter and Jernvall, 1995; Collard and Wood, 2000; Finarelli and Clyde, 2004). Additionally, by examining enamel thickness variation and its heritability in pedigreed baboon molars, Hlusko et al. (2004) showed that enamel thickness could change rapidly under moderate or low selective pressure over evolutionarily short periods, increasing the potential for homoplasy. Although the OES morphology is directly related

66 to dental functions such as occlusion and feeding and thus is a direct target of natural selection, the
67 morphology of EDJ has been considered to be more conservative evolutionally and a more reliable
68 representation of the phenotype for estimating phylogenetic relationships (Kraus, 1952; Korenhof,
69 1960; Smith et al., 1997; Sasaki and Kanazawa, 1999; Smith et al., 2000; Olejniczak et al., 2007).
70 So far researchers have explored to which extent enamel formation influences on the crown
71 morphology by comparing EDJ with OES (Kraus, 1952; Nager, 1960; Korenhof, 1960, 1961; Sakai
72 and Hanamura, 1971; Skinner et al., 2008, 2009; Ortiz et al., 2012). However, these studies mainly have
73 focused on discrete dental traits. Although a few studies tried to evaluate general morphological
74 difference between EDJ and OES quantitatively by using intercusp distance (Smith et al., 1997) or
75 surface complexity (Skinner et al., 2010), complex dental crown topography of EDJ and OES has not
76 been clarified in detail. Examining morphological variation and covariation between EDJ and OES
77 enables us to understand the effects of morphological change caused by enamel formation.
78 Additionally, given the different developmental backgrounds between the EDJ and OES, it is
79 likely that the patterns of phenotypic variability differ between these structures. Phenotypic variability is
80 defined as the tendency or potential of an organism to vary (Wagner and Altenberg, 1996; Wagner et al.,
81 1997; Willmore et al., 2007). Therefore, it determines the potential range or distribution of
82 morphological variation, and ultimately affects the tempo and mode of evolutionary change. The recent
83 literature about phenotypic variability has paid the greatest attention to canalization and morphological
84 integration (Wagner and Altenberg, 1996; Hallgimmson et al., 2002; Willmore et al., 2007;
85 Hallgrímsson et al., 2009). Canalization is generally considered a property of an organism that limits
86 phenotypic variation by buffering developmental processes against both environmental and genetic

perturbations (Wagner et al., 1997; Willmore et al., 2007). Morphological integration refers to the tendency for different characters to covary as a result of common underlying developmental factors (Hallgrímsson et al., 2002), which constrains the production of phenotypic variation (Wagner and Altenberg, 1996; Chernoff and Magwene, 1999). Canalization and morphological integration are interrelated and can act as developmental constraints (Alberch, 1982; Maynard Smith et al., 1985; Hallgrímsson et al., 2002). Since the morphological integration framework is directly connected to the rate and direction of evolutionary change (Cheverud, 1996; Wagner and Altenberg, 1996), some studies have focused on quantification of the intervening effect of morphological integration on evolutionary trajectory (Lande, 1979; Lande and Arnold, 1983). The resultant data have led to recent studies that evaluated evolvability (the ability of a population or species to respond to selection: Hansen, 2003) using the simulation of evolutionary responses to selection (Marroig et al., 2009; Villmoare et al., 2011; Lewton, 2012; Grabowski, 2013). The relationships and interactions among developmental processes, variability and variation, mediated by the feedback loop of natural selection, are critically involved in evolutionary change (Willmore et al., 2007). Comparison of the pattern of variability between EDJ and OES helps to infer how the production of morphological variation is regulated in each of these components.

In this study, we explore the relationship between the crown morphology and odontogenesis through quantitative analyses of the EDJ and OES morphology. We hypothesized that overall dental crown shape and covariation structure are determined by processes that configurate shape at the EDJ. If this hypothesis is rejected, a significant role of enamel formation for patterning of crown morphological variation must be presumed. To test this hypothesis, we described morphological variation and

covariation between EDJ and OES and revealed how much variation in the OES shape is explained by the EDJ shape variation. Consequently, we evaluated the strength of two components of phenotypic variability: canalization and morphological integration, in addition to the relevant evolutionary flexibility.

Canalization: if EDJ shows larger variation, it means that more variable morphology is created during the early phase of the tooth development, and subsequent enamel formation acts as stabilizing developmental process that buffers the deviation from mean shape. On the other hand, if OES shows larger variation, it indicates that enamel formation brings about not only homogeneous enamel distribution above the EDJ after the morphogenesis, but also some modification of the OES associated with the increased variation.

Morphological integration: if either during morphogenesis or the enamel formation process, some developmental factors produce higher morphological integration of one of these structures (whether EDJ or OES). Combined with the result regarding canalization, this analysis will help to determine what factors play important roles in generating or reducing morphological variance.

Evolutionary flexibility: in relation to the above two components of phenotypic variability, we specifically compared how the developmental constraints exert influence on the ability of the response to selection in EDJ versus in OES.

This study focused on EDJ and OES shape variation of maxillary permanent first molar (UM1) and second deciduous molar (um2). Although UM1 and um2 share similar patterns of occlusal morphology that are elaborated through the same developmental processes, their developmental timing, speed and period are different (Nanci, 2013). The differences between UM1 and um2 will provide a

129 better understanding of the relationship between odontogenesis and crown morphological variability.

130

131 **Materials and Methods**

132 The samples used in this study comprised fully formed but unworn UM1 and um2 crowns
133 obtained from archaeological sites in Japan. The total sample (57 UM1 and 48 um2) consisted of
134 samples from the Jomon (14500-300 BC; n=8 and 5), Medieval (13-15C AD; n=13 and 8), and Edo
135 (17-19C AD; n=36 and 35) periods. Although the total sample was from a mixture of populations from
136 different periods and regions, the aim of this study was to investigate differences and patterns of
137 variability produced by a common tooth formation process of the Holocene human, and mixing these
138 samples does not violate the objective of this study. In order to maximize sample size, no discrimination
139 between right and left teeth was made, but only a single tooth was used from each individual. All
140 specimens were regarded as left side. Right molar images were transformed into the mirror image using
141 ImageJ software (NIH, USA). Sex was unknown for most of the samples, since they were taken from
142 juvenile individuals.

143 Each specimen was μCT scanned (ScanXmateA080S, Comscantecno, Japan) with a pixel
144 size and slice interval of 31–32 μm (80 kV, 125 μA). To facilitate tissue segmentation, the image stack
145 for each tooth was filtered using a median filter followed by a kuwahara filter, and enamel and dentin
146 tissues were segmented by the seed region growing method in ImageJ. Triangular mesh models of the
147 3D EDJ and OES of each specimen were reconstructed using Analyze 6.0 (Mayo Clinic, USA) with the
148 marching cube method. Subsequent procedures were done using the software Rapidform 2004 (INUS
149 Technology, Korea).

We treated the EDJ and the OES as biologically corresponding structures in order to compare variability between them directly, and digitized (semi)landmarks on both of them in the same way as follows. We digitized main cusp tips (paracone, protocone, metacone, and hypocone) at the OES and the dentin horn tips at the EDJ, and the lowest points on the ridges at both the OES and the EDJ, connecting the two cusps as landmarks. Each ridge on both the OES and the EDJ was divided into eight sections by the cusp tips and the lowest points, respectively. For each section, a given number of semi-landmarks was digitized equi-distantly, as illustrated in Figure 1. The number of semi-landmarks on the EDJ and the OES were determined to satisfy two criteria, namely, that each corresponding section in the EDJ and the OES had the same number of (semi)landmarks, and that the contributions of the section between the (semi)landmarks to the curve were approximately equal to each other (Skinner et al., 2009; Skinner and Gunz, 2010). The dataset consisted of four configurations (UM1EDJ, UM1OES, um2EDJ and um2OES), and each of them had a total of 8 landmarks and 48 semi-landmarks.

Semi-landmarks are not considered to be homologous landmarks unless they are slid (Bookstein, 1997). The minimum bending energy algorithm (Bookstein, 1997; Gunz et al., 2005) was adopted. This data processing was performed by W. Y. using MATHEMATICA 8 (www. wolfram.com). Each homologous set of landmarks was converted to shape coordinates by Generalized Procrustes Analysis (GPA; Rohlf and Slice, 1990), which was performed using MorphoJ version 1.05d (Klingenberg, 2011).

Morphometric analysis

Covariation between EDJ and OES was analyzed using 2B-PLS. This method compares two morphological data sets by using a singular value decomposition of the cross-covariation matrix, finds new pairs of axes that account for the maximum amount of covariance between both data sets and visualizes the main associated morphological changes. The RV coefficient was used to evaluate the strength of multivariate correlations between data sets. This coefficient is a multivariate analogue of the squared correlation coefficient (Escoufier, 1973; Klingenberg, 2008). The significances of both the correlation between the scores for each pair of PLS axes and RV coefficient were evaluated by means of resampling tests with 1000 random permutations. These procedures were carried out with MorphoJ software (Klingenberg, 2011).

A principal component analysis of Procrustes shape coordinates was used to extract main patterns of morphological variation across EDJ and OES in both UM1 and um2. Using first few PC scores of EDJ and OES, we performed a regression analysis between these two structures to test whether shape variation of OES can be predicted by that of EDJ.

The difference in multivariate morphological change vector from EDJ to OES between UM1 and um2 was assessed by calculating the length and direction of shape change using a residual randomization procedure outlined in Collyer and Adams (2007). The length of a vector describes the overall amount of morphological change and the direction of a vector describes the way in which multiple traits covary. Observed vector lengths and directions were compared with 999 random permutations plus the observed value to assess significance.

Variability analysis

Among-individual phenotypic variation is the most common measurement of canalization. Canalization is generally inferred from a reduction of the observed phenotypic variance. Here we quantified both size and shape variance within each of the four configurations. For size, Centroid size (CS) of each configuration was calculated. Coefficient of variation (CV) of the LogCS was used to compare size variation, and tested as suggested by Sokal and Braumann (1980). For comparison of shape variability among configurations, the square root of the sum of the squared distances between Procrustes transformed coordinates of each cusp and its landmark mean configuration was used as the measure of shape variation. To test whether there was a significant difference of variability between the EDJ and the OES within the same tooth class, a nonparametric Kruskall-Wallis test and multiple-comparison test were performed.

To compare the overall strength of morphological integration, we followed Wagner (1984) in using the variance of the eigenvalues for the variance-covariance matrix as the measure of integration. This measure of integration captures whether shape variance can be explained by a small number of principal components, or whether variance is more evenly distributed across principal components. The former case would be considered more integrated and the latter less integrated. Variance of eigenvalues (VE) was compared between the EDJ and the OES within the same tooth using bootstrap resampling methods (Manly, 1997). For each of the EDJ and the OES, the original data matrix was bootstrapped 1000 times, a variance-covariance matrix was derived from each bootstrap sample, and VE was calculated from each of the 1000 variance-covariance matrices. For each of the 1000 VE replicates, the difference between the EDJ and the OES was calculated. This created a distribution of differences in VE replicates that was then zero-centered. Each of the zero-centered differences was then compared to

the observed difference in VE between the EDJ and theOES. The two-tailed P value was calculated as the number of times the difference from the zero-centered distribution was equal to or greater than the observed difference, divided by the number of bootstrap replicates (Manly, 1997).

The ability of EDJ and OES morphology to respond to selection was evaluated using mean flexibility (f) (Marroig et al. 2009), which is derived from Lande's (1979) multivariate selection equation:

$$\Delta z = G\beta$$

where G is the genetic covariance matrix, β is a selection vector, and Δz is the response vector. Here the phenotypic covariance matrix P is substituted for G because previous studies established structural similarity between them (e.g., Cheverud, 1996; Porto et al., 2009). The covariance matrix for each of EDJ and OES was subjected to 1,000 randomly generated selection vectors and the angle between the selection and response vectors was calculated for each time. The mean cosine of angles in 1000 repeats is called the mean flexibility (Marroig et al., 2009), which describes the degree to which the response and selection vectors are aligned in multivariate space. Response and selection vectors that are parallel (i.e., when the cosine of the angle between them is 1.0) indicate a structure that is more responsive to selection, i.e., more evolvable. A larger angle between the response and selection vectors is indicative of less evolvability. In general, high levels of evolvability measures, such as evolutionary flexibility, tend to be associated with low levels of integration measures (e.g., VE). Pairwise comparisons of evolutionary flexibility between EDJ and OES within the same tooth class were performed as described for VE; the distribution of vector correlations obtained from the covariance matrix and 1,000 random selection vectors for EDJ and OES were compared using the difference of means test and accompanied

by a two-tailed P value. All statistical analyses were performed using R version 2.13.1 (R Development Core Team, 2011).

Results

Morphometric analysis

Covariation between EDJ and OES is higher in um2 (RV=0.914; P<0.001) than in UM1 (RV=0.794; P<0.001). 2B-PLS analysis in UM1 revealed that the first axis explained 49.43% of total shape covariance and that corresponding shape change mainly involves the contraction of buccal side and expansion of distolingual cusp (hypocone) for both EDJ and OES (Table 1; Fig. 2a). The second axis also revealed that EDJ and OES showed similar shape change that contraction of mesiobuccal cusp (paracone) and contraction of distal side (Fig. 2b). In um2, the first singular axis of correspondence to the comparison of EDJ and OES revealed a correlated reduction of mesiolingual-distobuccally and expansion of mesiobuccal-distolingually (Fig. 2c). The second axis also revealed significant shape change of reduction of mesial cusps and reduction of distal cusps for both EDJ and OES (Fig. 2d).

In the PCA, the first two principal components account for 34.85% of the total variation (Figure 3a; Table 2). Positive scores of PC1 are associated with relatively high and sharp cusp tips and lingually located hypocone. Its negative values correspond to relatively-gentle and inner located cusp tips with deep intercuspal grooves. Positive PC2 scores are associated with mesial expansion and contraction of protocone and negative ones with mesial contraction with lingually expanded protocone. PC1 corresponds to the distinction between EDJ and OES, whereas PC2 separates between UM1 and um2. Figure 3b and 3c illustrates the regressions of first two PCs for EDJ and OES in both teeth. The

adjusted R- squared value is lower in UM1 than that in um2 for both PC1 (0.249 vs.0.700) and PC2 (0.842 vs. 0.907), which indicated that the OES shape variation is better predicted by EDJ shape variation in um2 than in UM1.

The tooth specific morphological change vectors between EDJ and OES were not statistically different in length (ΔD=0.004; P=0.27). However, the angle between these vectors was significantly greater than expected by chance (θ =27.62°; P<0.001: Fig. 3a).

Variability analysis

Canalization

The coefficients of variation of the LogCS for each configuration (UM1EDJ, UM1OES, um2EDJ and um2OES) was not significantly different from each other, although OES tended to be more variable than EDJ in both the UM1 and um2 tooth classes (Figure 4a). On the other hand, shape variability was significantly different among these configurations, and pair-wise tests showed that only in UM1 was there a significant difference in shape variability between EDJ and OES (Figure 4b).

Morphological integration

The variance of the eigenvalue (VE) was significantly greater for EDJ than for OES in UM1, but not in um2 (Figure 4c). The greater VEs for EDJ were seen in both UM1 and um2, indicating that EDJ was more integrated than OES.

Evolutionary flexibility

The mean cosine between the selection vector and the response vector for OES tended to be greater than that for EDJ, but a significant difference was not detected between them in either tooth class (Figure 4d). This meant that there was no difference in the extent to which EDJ and OES would be influenced by the selection vector.

Discussion

Both UM1 and um2 showed significantly correlated shape changes between EDJ and OES corresponding to singular axes. Enamel formation does not alter the basic morphology of the dentine horn and EDJ ridges and corresponding features (cusp tips and ridges) on OES. Our results accord with previous studies that dental traits seen in EDJ can be observed at OES (Korenhof, 1961, 1982; Nager, 1960; Sakai and Hanamura, 1973; Corruccini, 1998; Sasaki and Kanazawa, 1999; Skinner et al., 2008; 2009), which supports the major role of the EDJ in their origin and degree of dental crown traits. However, this does not necessarily mean that tooth shape and covariation structure are predetermined by processes that configurate tooth shape at EDJ. Comparisons between um2 and UM1 revealed different influences of enamel formation on the OES morphology. In um2, OES shape variation is better predicted from EDJ shape variation. Thus, multivariate covariation between EDJ and OES is higher compared to UM1. This result suggests that morphological change caused by enamel formation is more stable and less vulnerable to random perturbations in um2. This could be attributed to the difference in the enamel thickness (Grine, 2005), the rate of enamel formation (Shellis, 1984) and/or period of enamel formation (Liversidge and Molleson, 2004). While the amount of overall morphological change induced by enamel formation does not differ between UM1 and um2, the direction of change described

297 by traits covariation marks a significant difference. Given the different period of formation between
298 UM1 and um2 (Nanci, 2013), it may be expected that they show resembling directions of
299 morphological change with different amounts of morphological change. However, the result is converse,
300 suggesting a complex nature of crown enamel formation. For example, Grine (2005) noted that the
301 difference in enamel thickness between the paracone tip and the protocone tip was greater in um2 rather
302 than in UM1. The difference in patterns of enamel distribution between UM1 and um2 might affect the
303 way of covariation between EDJ and OES. Thus, enamel formation has a significant effect on *patterns*
304 of morphological change, probably according to tooth-specific developmental parameter, though it does
305 not cause a drastic change in morphology during odontogenesis.
306 The lack of significant difference in size variation between EDJ and OES in both tooth
307 classes examined here suggests that the strength of canalization on size is almost constant throughout
308 the processes of morphogenesis and the subsequent enamel formation period. A recent developmental
309 study revealed that molar crown sizes were regulated by intrinsic factors from mesenchymal tissues (Cai
310 et al., 2007) and adjacent molars during development (Kavanagh et al., 2007). Several dental metrics
311 studies confirmed that tooth crown size was less variable than intercusp distance and/or cusp size owing
312 to stronger genetic control (Townsend et al., 2003; Harris and Dihn, 2006), which would be also
313 supported by experimental evidence that cusp density (intercusp distances) was likely to be polygenic
314 (Harjunmaa et al., 2012). The present analysis of EDJ and OES at the dentin horns/cusp tips and ridges
315 provided the insight about intercusp distances that their size variation might not be altered largely by
316 enamel formation. Additionally, the spatial relationship with the surrounding tissues, including the
317 maxillary bone and/or other tooth germs, and the available space for tooth growth (Boughner, 2011)

318 may be involved in the canalization of crown size during odontogenesis. The extent of the deviation
319 from mean size in EDJ and OES were not significantly different, and therefore both EDJ size
320 differences and OES size differences among groups being compared can be used as a reliable measure
321 of phylogenetic relatedness.

322 In the case of UM1, shape variation of OES was greater than that of EDJ. This result
323 suggests that canalization of crown shape may be weakened during the process of enamel formation.
324 Kraus and Jordan (1965) argued that early stages of tooth development were mediated by genes that are
325 more evolutionarily stable than those associated with calcification. Hlusko's (2004) simulation model
326 indicated that enamel thickness could change rapidly under appropriate selective pressure. The present
327 result obtained at the cusp tips and ridges is in accord with these studies and implies that shape (e.g.,
328 intercusp topological relationship) variation is more susceptible to modifications resulting from enamel
329 formation than size variation, which might be likely to cause homoplasy that would confuse
330 phylogenetic reconstructions (note here "size" refers to the centroid size of the cuspal tips and ridges and
331 not commonly used crown size proxies like maximum mesiodistal x buccolingual dimensions).

332 The result of VE analysis showed that EDJ was more integrated than OES in UM1, although
333 the same was not supported statistically in um2. Molar crown morphogenesis is a morphodynamic
334 process in which inductive events and morphogenetic processes act at the same time, and is regulated by
335 interactions between the epithelial and underlying mesenchymal tissues. Cusp initiation and patterning
336 in tooth germ is an iterative process that repeatedly utilizes the same set of genes and signaling pathways,
337 which would lead to higher morphological integration in EDJ. On the other hand, the pattern of enamel
338 formation is the end product of a sequence proceeding from ameloblast differentiation from the IEE

339 cells, to secretion of enamel proteins including amelogenins and enamelins, and finally organization of
340 the enamel crystallites into enamel rods or prisms (Boyde, 1964, 1989). Topological developmental
341 parameters, such as the rate and the duration of enamel apposition and/or ameloblast extension and
342 termination (Simmer et al., 2010), might impact the OES formation, which could lead to weaker
343 morphological integration in OES.
344 It is predicted that stronger integration between traits acts as a limitation on producing
345 phenotypic variation (Wagner and Altenberg, 1996). The results of the canalization and morphological
346 integration analyses presented here are consistent with this prediction, i.e., the more strongly integrated
347 EDJ shows smaller variability. The set of genes expressed during morphogenesis of the tooth are also
348 used in different organs, including hair, pancreas, mammary gland, salivary gland, thymus, vibrissae,
349 and others (Fincham et al., 2000; Jernvall and Jung, 2000). Mutations in coding region that alter the
350 function or activity of proteins are likely to have widespread and potentially many negative effects on
351 development and fitness, and may thus be under considerable constraint (Carroll, 2008). Size and shape
352 of EDJ are thus more likely to be stabilized in order to reduce the risks of negative pleiotropic side
353 effects. The high level of integration in EDJ can be regarded as a relatively rigorous developmental
354 constraint during odontogenesis. Meanwhile, the set of genes that contribute to enamel formation, such
355 as amelogenin, enamelin, ameloblastin, and enamelysin genes, is highly specialized, and can easily
356 modify the OES morphology during the enamel formation process. Morphological change of the OES,
357 which has less developmental constraint, can easily be brought about by neutral evolution by
358 non-natural selective genetic factors such as random genetic drift.
359 The observed pattern of morphological integration and the results of evolutionary flexibility

360 analyses presented here are not consistent with those of previous studies, in which low levels of
361 integration accompanied high levels of evolvability (Marroig et al., 2009; Porto et al., 2009; Lewton,
362 2012). The developmental constraints due to canalization and morphological integration act more
363 strongly on the shape of EDJ than on that of OES in UM1, while there is no significant difference in the
364 evolutionary flexibility between EDJ and OES. This may result from the relatively integrated
365 covariance structure of each cusp (for both EDJ and OES). Since the secondary enamel knot that
366 functions as a signaling center and regulates cusp formation at the future cusp tip acts as a
367 "developmental module" (Jernvall and Jung, 2000), it can directly affect the covariance structure of EDJ,
368 and indirectly affect that of the overlying OES. In the case of the human tooth, if the crown covariance
369 structure is divided into individual cusp units, this patterning cascade mode of cusp development
370 facilitates the ability to respond to selective challenges (Jernvall and Jung, 2000), and enables the
371 maintenance of a certain level of evolvability at EDJ despite existence of developmental constraints.
372 The comparable level of evolutionary flexibility between EDJ and OES suggests that both of them can
373 be utilized as an equally effective proxy for inferring phylogenetic relationships that would result from
374 selective pressure.

375 Overall, the difference of each measurement (canalization, morphological integration and
376 evolutionary flexibility) between the EDJ and OES in the present study was greater in UM1 than in um2.
377 The process of enamel formation is more likely to influence crown morphological variability and
378 evolvability in UM1 than in um2, which can be explained by the duration and/or thickness of enamel
379 formation. Compared to UM1, the enamel deposition period of um2 is shorter and the enamel is thinner
380 (Nanci, 2013). Therefore enamel formation may exert less influence on shape change in um2, which

may be related to the conservation of primitive morphology, as discussed in previous studies (Dahlberg, 1945; Butler, 1956, 1971; Suzuki and Sakai, 1973; Saunders and Mayhall, 1982). Since not only morphology but also variability would be likely to differ between EDJ and OES, a tooth crown that has a longer period of enamel formation and/or thicker enamel would require careful evaluation for phylogenetic studies.

This study compared patterns of canalization, morphological integration, and evolutionary flexibility between the EDJ and the OES in UM1 and um2 in order to explore their possible effects on phylogenetic reconstructions. Our results suggest that a tooth crown that has thicker enamel and/or a longer period of enamel formation can be more variable in shape at the OES, where similarity can be due to homoplasy. Recent advances in imaging techniques have made it possible to approach the details of developmental trajectories reflected in the teeth of fossil species (Avishai et al., 2004; Smith et al., 2011). Understanding the morphological variability and evolvability produced by the developmental process is an important step in validating phylogenetic hypotheses based on the OES morphology alone.

Concluding Remarks

Both morphometric and variability analyses indicate that tooth shape and covariation structure is not only determined by processes that contribute to tooth shape at the EDJ, but also amelogenesis can play a significant role in them. The influence of enamel formation on morphological variation and patterns of variability is not constant among teeth, which may be responsible for the differences in the rate and/or period of enamel formation.

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Author contributions

W.M. and W.Y. designed the research and performed the analysis. W.M., T.N., and M.A. collected the data. W.M., H.O., and M.N. wrote the manuscript.

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581 **Tables**

Table 1 Results of PLS analyses between EDJ and OES corresponding to UM1 and um2

	UM1			um2		
	% Total Cov.	Correlation coefficient	P-value[1]	% Total Cov.	Correlation coefficient	P-value[1]
1	49.43	0.951	<0.001	43.14	0.974	<0.001
2	17.39	0.933	<0.001	25.11	0.970	<0.001
3	14.65	0.908	<0.001	17.76	0.954	<0.001
4	10.22	0.879	<0.001	6.52	0.948	<0.001

[1]Randomiztion rounds: 1000

582

Table 2 Results of principal component analysis with the total sample

	Eigenvalue	% Explained variance	% Cumulative variance
1	0.0016	19.99	19.99
2	0.0012	14.86	34.85
3	0.0009	11.80	46.64
4	0.0007	9.08	55.73
5	0.0005	6.86	62.58
6	0.0005	6.68	69.26
7	0.0004	5.31	74.58
8	0.0002	3.14	77.71
9	0.0002	2.90	80.61
10	0.0002	2.35	82.96

583

584 **Figure legends**

585 Figure 1. Digital image of permanent maxillary first molar crown (lingual view). (a) EDJ ridge curve
586 digitized on the EDJ surface. (b) OES ridge curve digitized on the OES. Red circles are landmarks, and
587 yellow circles are semi-landmarks. Numbers appended to each section of the ridge curve refer to the
588 equally-spaced interpolated semi-landmarks.

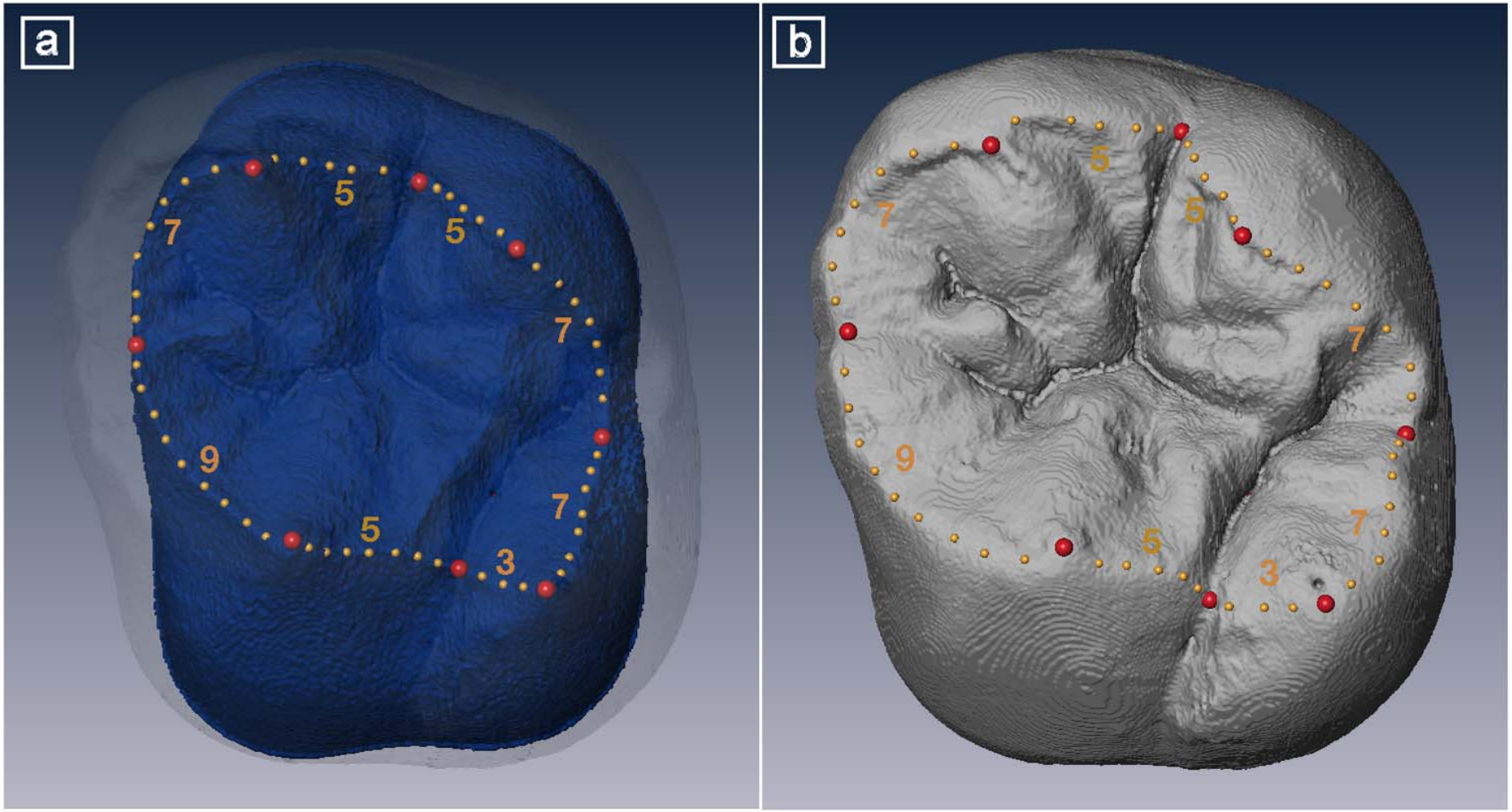
589

590 Figure 2. Scatter plots representing the first and second pairs of PLS axes between EDJ and OES within
591 the same tooth class. (a) PLS1 UM1, (b) PLS2 UM1, (c) PLS1 um2, (d) PLS2 um2. Shape deformation
592 corresponding to each axis is provided to the left of x-axes or above y-axes. Each shape deformation is
593 represented in colored line whose scale factor used for is 0.1 and mean shape is represented in gray line.

594

595 Figure 3. Principal component plots for shape variation between EDJ and OES of both UM1 and um2.
596 (a) Plots of PC1 versus PC2 scores. Variance explained by PC1 and PC2 is 34.85% of total variance.
597 Shape deformation corresponding to the positive or negative loadings of each axis is provided to the left
598 and right for x-axes or the above and bottom for y-axes. Each shape deformation is represented in
599 colored line whose scale factor used for is 0.1 and mean shape is represented in gray line. Arrows show
600 morphological change vectors from mean shape represented in large symbols of EDJ to that of OES for
601 each tooth class. (b) Relationship between EDJ and OES for PC1 in both UM1 and um2. The slope and
602 intercept of the regression line for UM1 are 0.804 and -0.070, respectively ($r=0.51$, $P<0.001$). The slope
603 and intercept of the regression line for um2 are 0.876 and -0.068, respectively ($r=0.84$, $P<0.001$). (c)
604 Relationship between EDJ and OES for PC2 in both UM1 and um2. The slope and intercept of the

605 regression line for UM1 are 0.863 and -0.002, respectively ($r=0.92$, $P<0.001$). The slope and intercept of
606 the regression line for um2 are 0.918 and 0.007, respectively ($r=0.95$, $P<0.001$).
607
608 Figure 4. (a) Bar graph showing the size variation for four configurations (UM1EDJ, UM1OES,
609 um2EDJ and um2OES). Significance test for coefficient of variation for LogCS among them reveals
610 that there is no significant difference ($P>0.05$). (b) Bar graph showing mean of propcrustes distance
611 from each mean shape for shape variance of four configurations (UM1EDJ, UM1OES, um2EDJ and
612 um2OES), and the error bars show standard deviations. The Kruskall-Wallis test reveals a significant
613 difference among them ($P<0.001$). A nonparametric multiple-comparison test between EDJ and OES
614 within the same tooth class reveals that the difference is highly significant in UM1 ($P<0.001$). (c) Bar
615 graph showing the scaled variances of eigenvalue for morphological integration for four configurations
616 (UM1EDJ, UM1OES, um2EDJ and um2OES). The error bars shown are standard deviations obtained
617 by resampling the original datasets with replacement 1000 times. Bootstrap tests between EDJ and OES
618 within the same tooth class reveal that the difference is highly significant only in UM1 ($P=0.009$). (d)
619 Bar graph showing the evolutionary flexibility for four configurations (UM1EDJ, UM1OES, um2EDJ
620 and um2OES). The error bars shown are standard deviations obtained by resampling the original
621 datasets with replacement 1000 times. Bootstrap tests between EDJ and OES within the same tooth
622 class reveal that there is no significant difference ($P>0.05$).


a
7
5
5
7
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7
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3
b
5
7
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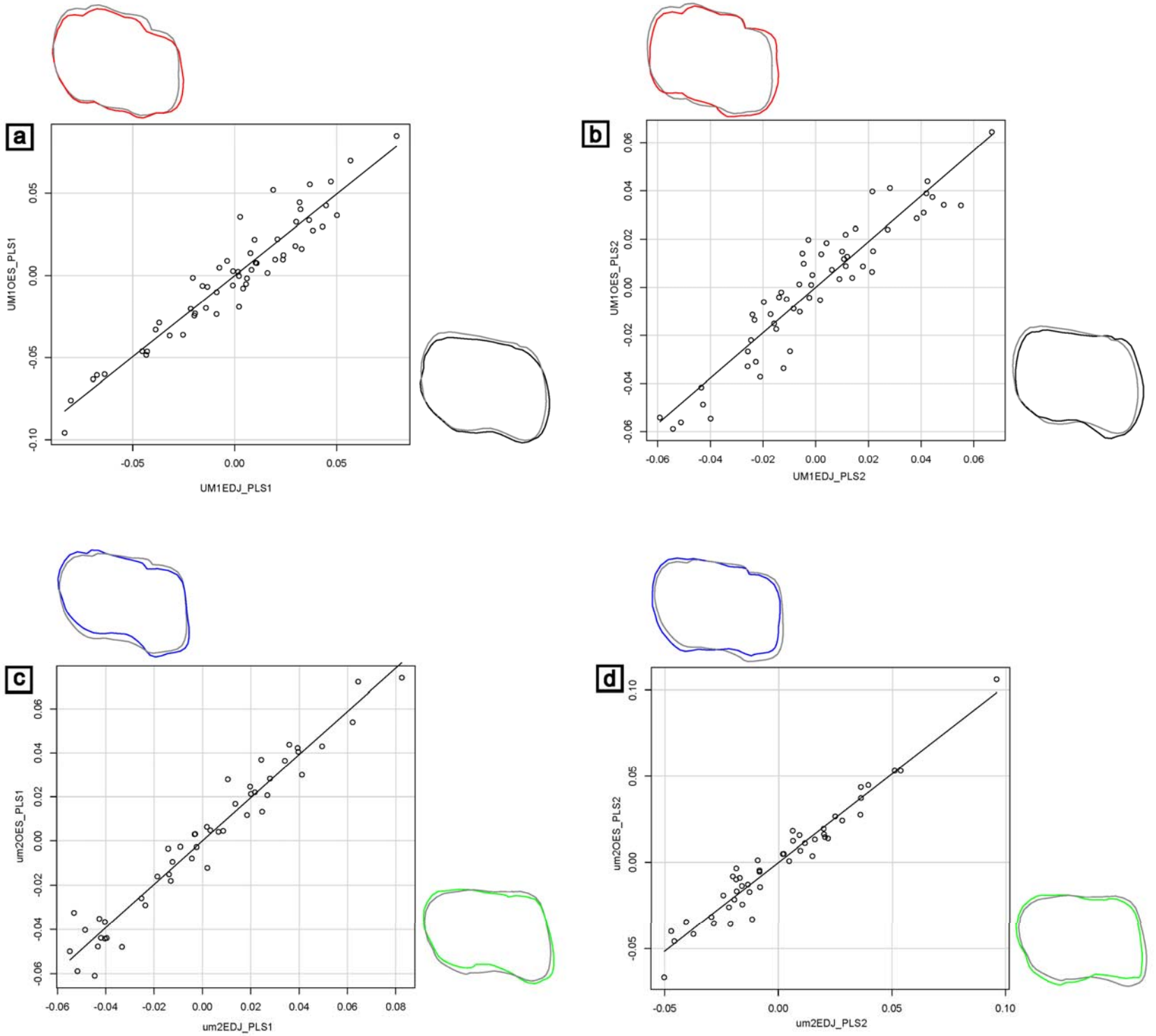
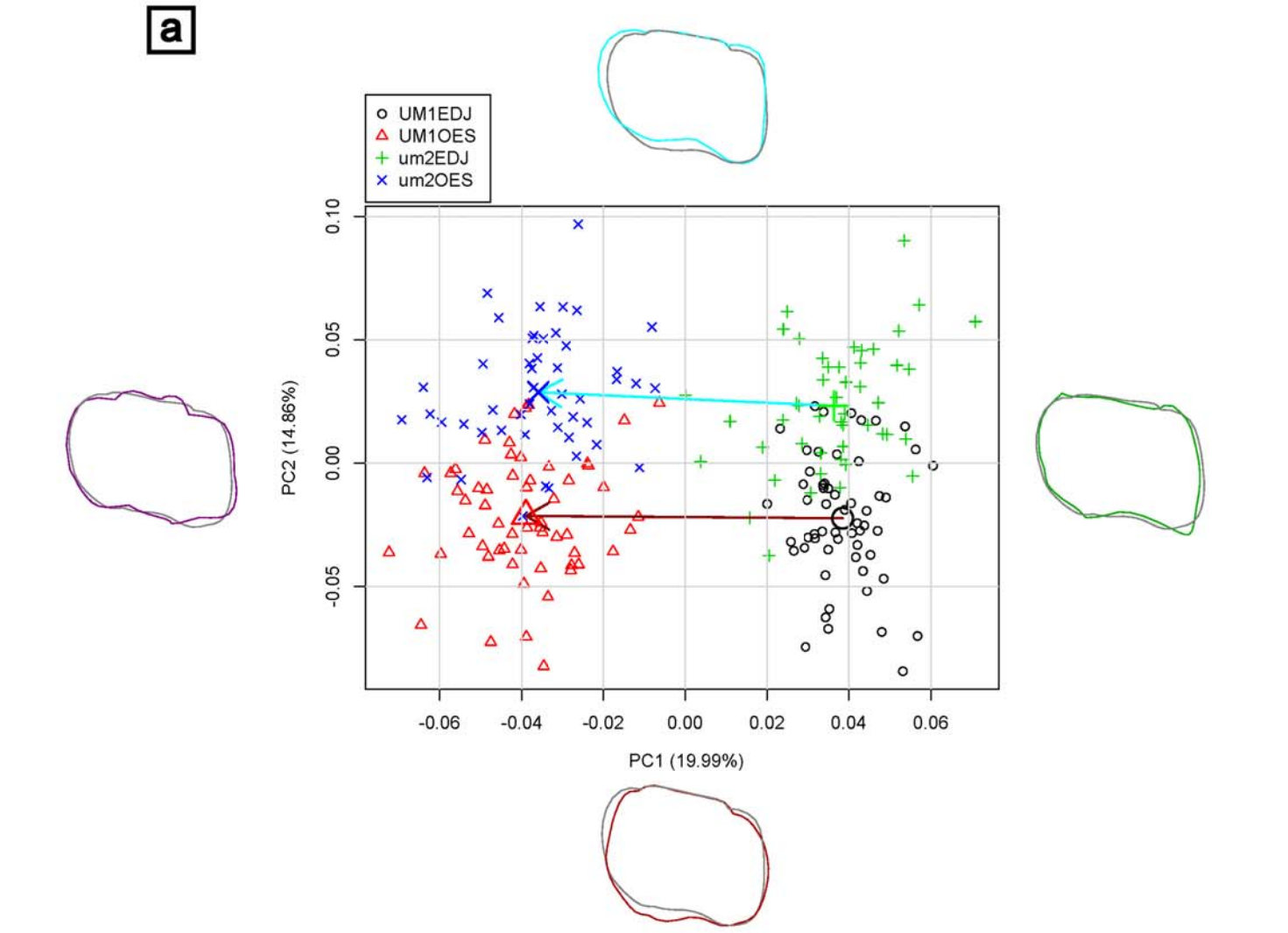
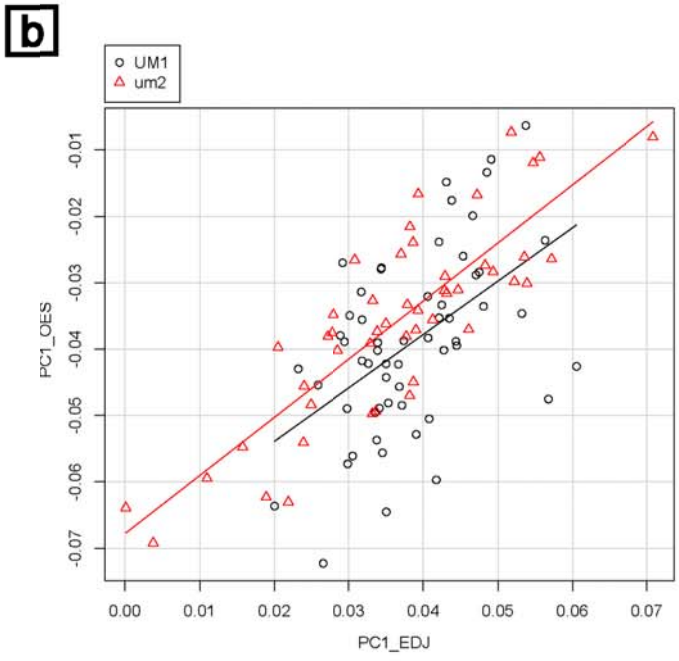
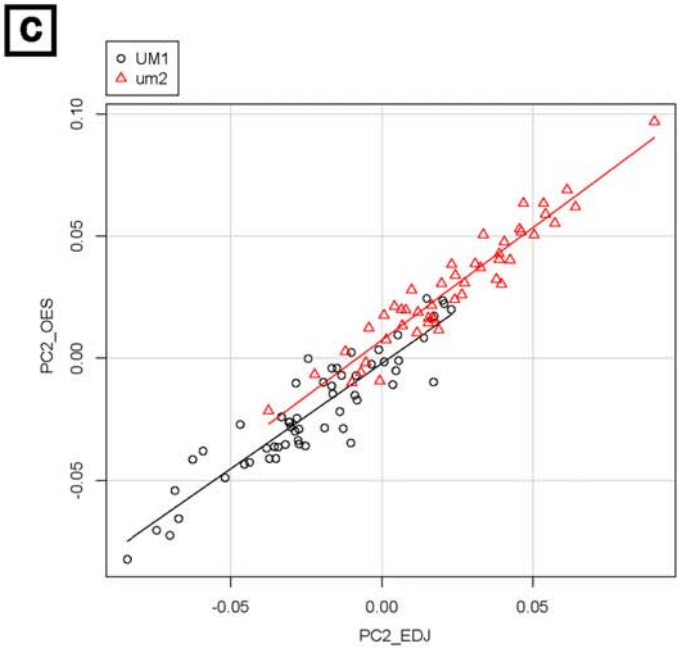
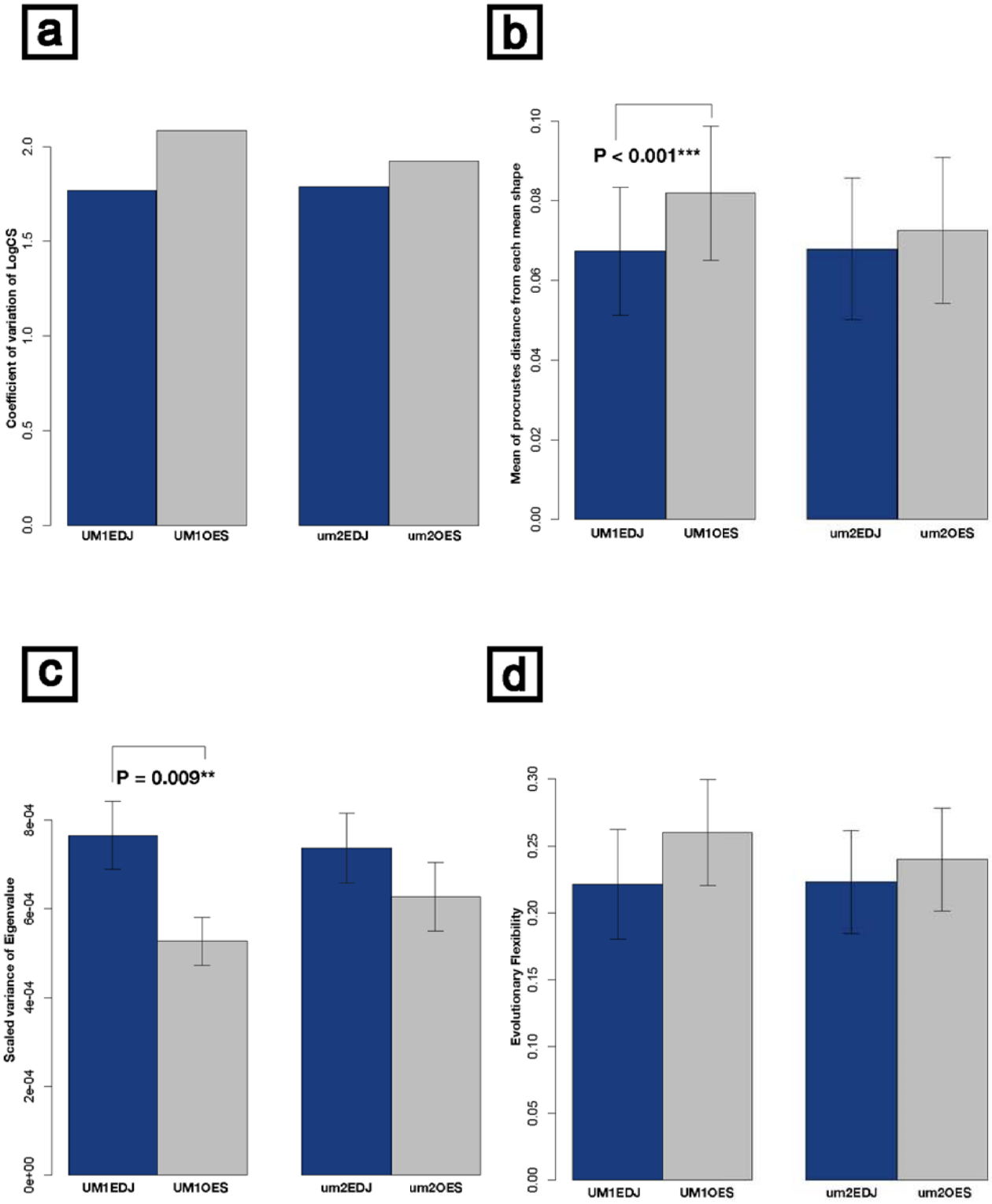
7
5
3


a
UM1OES_PLS1
UM1EDJ_PLS1
b
UM1OES_PLS2
UM1EDJ_PLS2
c
um2OES_PLS1
um2EDJ_PLS1
d
um2OES_PLS2
um2EDJ_PLS2


a
UM1EDJ
UM1OES
um2EDJ
um2OES
PC2 (14.86%)
PC1 (19.99%)
0.10
0.05
0.00
-0.05
-0.06
-0.04
-0.02
0.00
0.02
0.04
0.06


b
UM1
um2
PC1_OES
PC1_EDJ
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
-0.07
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07


c
UM1
um2
PC2_OES
PC2_EDJ
0.10
0.05
0.00
-0.05
-0.05
0.00
0.05


a
Coefficient of variation of LogCS
0.0
0.5
1.0
1.5
2.0
UM1EDJ
UM1OES
um2EDJ
um2OES
b
Mean of procrustes distance from each mean shape
0.00
0.02
0.04
0.06
0.08
0.10
P < 0.001***
UM1EDJ
UM1OES
um2EDJ
um2OES
c
Scaled variance of Eigenvalue
0e+00
2e-04
4e-04
6e-04
8e-04
P = 0.009**
UM1EDJ
UM1OES
um2EDJ
um2OES
d
Evolutionary Flexibility
0.00
0.05
0.10
0.15
0.20
0.25
0.30
UM1EDJ
UM1OES
um2EDJ
um2OES