

Morphological variability in human maxillary molars

Wataru MORITA

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By

Wataru MORITA

Graduate School of Science

Kyoto University

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Abstract

Introduction

Owing to the durable material nature and relatively compact size, teeth are well represented in the fossil record of mammals. Consequently, dental remains play a crucial role for paleo-anthropological/paleontological studies. Besides the taxonomic/phylogenetic studies, since dental developmental mechanism is well understood and its developmental process is recorded in fully formed tooth, tooth is a good subject for understanding relationship between development and morphological variation, which leads to morphological evolution. This study used geometric morphometrics for rigorous quantification of morphological variation of human molariform teeth, specifically focusing on the phenotypic variability. The principal aim of this dissertation is to understand morphological variability of human maxillary molar and to tackle following two specific questions:

- I. *How the patterns of morphological variability are structured during human molar odontogenesis?* Patterns of size and shape variability among cusps were examined at total and each particular crown components. Under inspired by developmental genetic studies, the prediction that the location and the size of later-forming cusps are more variable than those of earlier-forming ones based on the patterning cascade model (PCM) of cusp development was assessed.
- II. *What types of properties of morphological variability exists through the odontogenetic process, and how they affect evolutionary morphological change?* The strength of two components of phenotypic variability: canalization and morphological integration, plus the relevant evolutionary flexibility were compared between enamel-dentin junction (EDJ) and outer-enamel surface (OES) and explored the effects on reconstruction of the phylogenetic relationships.

Materials and Methods

Unworn maxillary permanent first molar (UM1) and second deciduous molar (um2) excavated from Japanese archaeological sites were used. Specimens were μ CT-scanned, and 3D models of EDJ and OES were reconstructed. For the first question, 3D (semi) landmarks

were digitized on each main cusp (paracone, protocone, metacone, and hypocone) region of four crown components (EDJ-ridge, OES-ridge, OES-circumferences, and CEJ) in a tooth. Using geometric morphometric methods, size and shape variation were compared among cusps. For the second question, I conducted the following analysis. Based on these models, landmark-based 3D geometric morphometric analyses were conducted. Individual phenotypic variation is used as the measurement of canalization of size and shape. To compare overall strength of morphological integration, we calculated the variance of the eigenvalues for the variance-covariance matrix for each four configuration. Evolutionary flexibility is evaluated by the mean cosine of angles between randomly generated selection vectors and the corresponding response vectors.

Results and Discussions

Size variability in both tooth types was generally consistent with the prediction yielded from the PCM, and the differences in size variation among cusps were smaller for the crown components that are completed in later stages of odontogenesis. However, regarding shape variability, the prediction was mostly unsupported, and UM1 and um2 showed different patterns. Our findings suggested that the pattern of size variability would be caused by temporal factors such as the order of cusp initiation and the duration from the beginning of mineralization to the completion of crown formation, whereas shape variability may be affected by both topographic and temporal factors.

The lack of a significant difference in size variation between EDJ and OES suggested that the strength of canalization on size was almost constant throughout odontogenesis. In UM1, EDJ showed less shape variation and a higher level of morphological integration than OES, which indicated that canalization and morphological integration acted as developmental constraints. In um2, such a tendency appeared weaker, probably due to the thinner enamel and/or shorter period of enamel formation. Evolutionary flexibility was not significantly different between EDJ and OES in either tooth, possibly because the PCM of cusp development would cause a cusp-divided covariance structure. This crown formation would lead to the retention of a certain level of evolvability of EDJ despite the existence of

developmental constraints.

In conclusion, the results of this study provided new information on the patterns and properties of morphological variability structured during odontogenesis in human molar, and its effect on evolutionary change, pointing the necessity of studies that elucidate the mechanisms of tooth morphological evolution.

Chapter 1: Introduction

Teeth are a vital element for the adaptation to feeding behavior and mastication, and strongly affect individual's fitness. Therefore, selection favors those dental variants with better functional occlusion, and greater durability, which would lead to a greater reproductive success.

Because of the durable material properties of enamel and dentin of the tooth crown, dental remains are well represented in the fossil and archaeological sites. Quantitative and qualitative analyses have demonstrated that the external morphology of the enamel can readily distinguish taxonomic groups, reconstruct the evolutionary relationships, and explore patterns of human migration (Suwa et al., 1994, 1996, 2007; Scott and Turner, 1997; Dean, 2000; Pilbrow, 2006; Bailey and Wood, 2007; Hanihara, 2008; Skinner et al., 2009; Gomez-Robles et al., 2007, 2011a, b).

Tooth morphology provides an advantageous model for seeking the developmental process that governs the production of the phenotypic variation. Tooth development is largely independent of the rest of the body. Unlike bones, teeth never experience remodeling under physiological conditions once their formation is completed. Because dental morphology is built only once during odontogenesis and unchangeable through the lifetime, it bears directly on questions concerning morphology and developmental process.

Moreover, tooth development is well understood through the histological and developmental genetic analyses of tooth germs. The tooth development is initiated as invagination of the oral epithelium (dental lamina) that are rapidly surrounded by the condensing neural-crest-derived mesenchyme (ectomesenchyme) in the bud stage (Kollar and Lumsden, 1979; Lumsden, 1987, 1988), leading to differentiation of the enamel organ and the dental papilla and follicle in the next stage. During the cap stage, the enamel organ extends further into the ectomesenchyme and wraps the condensing dental papilla in itself. Species-specific cusp patterns are configured in the subsequent bell stage, when the inner enamel epithelial cells, the part of enamel organ, differentiate into enamel-producing ameloblasts and the subjacent dental papilla cells differentiate into dentin-producing odontoblasts, resulting in the establishment of the final tooth configuration. Thus, the crown features are determined

during the cap and bell stages. The molecular basis regulating tooth development has been also studied extensively over the past decade (Thesleff, 2003; Zhang et al., 2005; Tummers and Thesleff, 2009). These studies have confirmed that the morphogenesis of tooth cusps is controlled by transient epithelial clusters, which are called enamel knots, using signaling molecules mostly belonging to the Wnt, Bmp, Fgf, and Shh families and are used similarly in the development of other organs (Jernvall et al., 1994; Vaahtokari et al., 1996; Jernvall and Jung, 2000). A primary enamel knot controls the initial folding of the inner enamel epithelium and is involved in the induction of several secondary enamel knots (Cho et al., 2007). The secondary enamel knots are non-proliferative signaling centers that control the nested expression of activator and inhibitor proteins in the dental epithelium and mesenchyme. In multicuspidate, secondary enamel knots appear sequentially at the future location of each cusp and repeatedly uses the same signaling pathways. The spatial patterning of cusps in the tooth germ is not predetermined, but rather the size, spacing and number of cusps are influenced by those of earlier-forming ones (Salazar-Ciudad and Jernvall, 2002). This morphodynamic process in which inductive events and morphogenetic processes act at the same time, has been called the Patterning Cascade Model (PCM) of cusp development (Jernvall, 2000; Jernvall and Jung, 2000). Under this model, small changes early in the developmental process can cause large difference in the fully formed tooth. Thus, there is a close relationship between the final crown morphology and the developmental events such as activation/deactivation of the enamel knot, mitotic activity in the inner enamel epithelium and cervical loop, and commencement/cessation of the mineralization, and so on (Lumsden, 1987; Jernvall, 1995). The variation of crown morphology is explained by differences in the relative timing of the sequential events occurring in the tooth developmental process. Thus, traits of the developmental patterns can be inferred, at least in part, from the fully formed teeth.

The odontogenesis consists of two important processes: the determination of basement membrane location and the deposition of dentin and enamel matrices proceeding from cusp tip to cervix of the tooth (Skinner et al., 2010). During tooth crown formation, the determined location of the basement membrane is preserved as the enamel-dentin junction (EDJ) in the developed tooth crown. The deposition of dentin proceeds inward and the enamel

is formed outward from the location of the basement membrane. Thus, the analyzable tooth configuration is the direct observable outer enamel surface in the fully formed teeth in the previous studies (Butler, 1956; Kraus and Jordan, 1965; Jernvall and Jung, 2000; Jernvall and Thesleff, 2000; Cobourne and Sharpe, 2003; Tafforeau et al., 2007).

However, it is difficult to reveal precise relationships between fully formed tooth morphological variation and developmental process in human teeth. Firstly, since the outer enamel surface in erupted teeth experiences morphological changes such as attrition, abrasion, caries, and artificial changes, it is difficult to analyze the precise dental morphology. Secondary, though the recent availability of large-scale data on human genome diversity has allowed to identify candidate genes for population-specific phenotypes (Kimura et al., 2009), an experimental approach cannot be directly applied to the study of fossil and extant humans.

This dissertation examined intact morphology that is formed through the developmental process in unworn maxillary permanent first molar (UM1) and second deciduous molar (um2). These two teeth have similar main-cusp and occlusal groove patterns and belong to the same molarization field (Butler, 1967), which does not contradict recent findings of molecular, cellular or genetic studies (Sharpe, 1995; Mitsidis and Smith, 2006). Although UM1 and um2 share similar patterns of occlusal morphology that are elaborated through the same developmental processes, their size, developmental timing, speed and period are different (Ten Cate, 2012). The crown of um2 is smaller than that of UM1, and is initiated 12.5-19 weeks after fertilization and is completed by 11 months after birth, whereas UM1 crown begins to calcify at birth and is completed at 2.6-2.7 years (Ten Cate, 2012). The meristic variation shown along the molar tooth row of modern humans, whose developmental pattern is well established, will provide a better understanding of the relationship between odontogenesis and crown morphology.

These specimens were scanned by high-resolution micro-CT, which allowed surface reconstructions of both the EDJ and outer-enamel surface (OES) to be generated that are able to quantify morphological variation of the complex human dental crown in 3D. Not only the morphological variation of OES, but also that of EDJ that represents the form of basement membrane has been studied by several ways. The chemical removal of overlying enamel was

employed due to a reasonable access to the EDJ morphology (Kraus, 1952; Nager, 1960; Sakai and Hanamura, 1971, 1973; Corruccini, 1987; Sasaki and Kanazawa, 1999), but it prevented further analyses of enamel and suffered from potential damage to the EDJ itself during removal procedure. Korenhof (1960) used the plaster endocasts obtained from enamel caps as proxies for EDJ, but it could not reliably document the presence or absence of fine details of discrete features. Computer-Tomography (CT) enables non-destructive study of internal structures and provides both 2D cross-section and 3D volumetric data. CT has been employed focusing on enamel thickness/distribution in dental morphometric studies (Schwartz, 2000; Hlusko et al., 2004; Grine, 2005). More recent analyses using high-resolution microCT addressed quantitative and/or qualitative evaluations of EDJ morphological variation (Smith et al., 2006; Olejniczak et al., 2007; Skinner et al., 2008a, b, 2009; Hunter et al., 2010; Ortiz et al., 2012), there are only a few studies on overall dental crown variation including both EDJ and OES morphology (Avishai et al., 2004; Skinner et al., 2010; Smith et al., 2007, 2011).

Crown morphological variation has been quantified by various ways, such as mesio-distal and bucco-lingual diameters, cusp dimensions, inter-cusp distance, and the number of surface orientation patches (Yamada and Brown, 1988; Hillson, 1996; Kondo et al., 1996; Skinner et al., 2009; Gomez-Robles et al., 2011a). However, these methodologies cannot capture complex morphological variation in dental crown sufficiently. Geometric morphometric methods that combine a geometric concept of shape with the procedures of multivariate statistics provide powerful and flexible set of tools for analyzing morphological variation (Bookstein, 1991; Dryden and Merida, 1998; Klingenberg, 2002) and are used in this study to quantify the size and shape of dental crown.

To elucidate the relationship between morphological variation and developmental process, which involved in morphological evolution, this dissertation focused on the concept of phenotypic variability with which connects developmental process, variation, and natural selection (Hallgimsson et al., 2002; Willmore et al., 2007). Phenotypic variability is defined as the tendency or potential of an organism to vary (Wagner and Altenberg, 1996; Wagner et al., 1997; Willmore et al., 2007). Developmental process structures the production of variation by modulating the amount of phenotypic variation and biasing of the distribution of the variants

that are produced, which involves variability. Expressed phenotypic variation in a population is what is available to natural selection and this biases the developmental processes available for subsequent generations. Thus, variability can have important effects on the rate and direction of evolutionary change.

The principal aim of this dissertation is to understand morphological variability of human maxillary molar. The data used to achieve this aim are digital reconstructions of the EDJ and the OES of human maxillary permanent first molar and second deciduous molar teeth acquired non-destructively through micro-computed tomography. These reconstructed models are analyzed quantitatively using geometric morphometric methods in the light of the following specific questions:

- I. *How the patterns of morphological variability in human molar teeth are structured during odontogenesis?* Under the PCM of cusp development inspired by developmental genetic studies, it is predicted that the location and the size of later-forming cusps are more variable than those of earlier-forming ones. This question was addressed by testing whether differences in the variability among cusps at total and each particular crown components [EDJ, OES, and cement-enamel junction (CEJ)] could be explained by the PCM.
- II. *What kinds of the properties of morphological variability structured by odontogenetic process are existed in human molar teeth, and how they affect evolutionary change?* This question was addressed through the quantification of two widely studied properties of variability: canalization and morphological integration, plus the relevant evolutionary flexibility, i.e., the ability to respond to selective pressure. These data also provided insights into the effects on reconstruction of the phylogenetic relationships.

References

- Avishai G, Müller R, Gabet Y, Bab I, Zilberman U, Smith P (2004) New approach to quantifying developmental variation in the dentition using serial microtomographic imaging. *Microsc Res Tech* 65, 263–299.
- Bailey SE, Wood BA (2007) Trends in postcanine occlusal morphology within the hominin

clade: The case of *Paranthropus*. In: *Dental Perspectives on Human Evolution: State of the Art Research in Dental Paleoanthropology* (eds. Bailey SE, Hublin JJ), pp. 33–52. New York: Springer.

Bookstein FL (1991) *Morphometric tools for landmark data: Geometry and Biology*. New York: Cambridge University Press.

Butler PM (1956) The ontogeny of molar pattern. *Biol Rev* 31, 30–70.

Butler PM (1967) Comparison of the development of the second deciduous molar and first permanent molar in man. *Arch Oral Biol* 12, 1245–1260.

Cho SW, Lee HA, Cai J, Lee MJ, Kim JY, Ohshima H, et al. (2007) The primary enamel knot determines the position of the first buccal cusp in developing mice molars. *Differentiation* 75, 441–451.

Cobourne MT, Sharpe PT (2003) Tooth and jaw: molecular mechanisms of patterning in the first branchial arch. *Arch Oral Biol* 48, 1–14.

Corruccini RS (1987) The dentinoenamel junction in primates. *Int J Primatol* 8, 99–114.

Dean MC (2000) Progress in understanding hominoid dental development. *J Anat* 197, 77–101.

Dryden I, Mardia KV (1998) *Statistical Shape Analysis*. New York: John Wiley & Sons.

Gómez-Robles A, Martínón-Torres M, Bermúdez de Castro JM, Margvelashvili A, Bastir M, Arsuaga JL, et al. (2007) A geometric morphometric analysis of hominin upper first molar shape. *J Hum Evol* 53, 272–285.

Gómez-Robles A, Martínón-Torres M, Bermúdez de Castro JM, Prado-Simón L, Arsuaga JL (2011a) A geometric morphometric analysis of hominin upper premolars. Shape variation and morphological integration. *J Hum Evol* 61, 688–702.

Gómez-Robles A, Olejniczak AJ, Martínón-Torres M, Prado-Simón L, Bermúdez de Castro JM (2011b) Evolutionary novelties and losses in geometric morphometrics: a practical approach through hominin molar morphology. *Evolution* 65, 1772–1790.

Grine FE (2005) Enamel thickness of deciduous and permanent molars in modern *Homo sapiens*. *Am J Phys Anthropol* 126, 14–31.

Hallgrímsson B, Willmore K, Hall BK (2002) Canalization, developmental stability, and

morphological integration in primate limbs. *Am J Phys Anthropol* 35, 131–158.

Hanihara T (2008) Morphological variation of major human populations based on nonmetric dental traits. *Am J Phys Anthropol* 136, 169–182.

Hillson S (1996) Dental anthropology. Cambridge: Cambridge University Press.

Hlusko LJ, Suwa G, Kono R, Mahaney MC. (2004) Genetics and the evolution of primate enamel thickness: A baboon model. *Am J Phys Anthropol* 124, 223–233.

Hunter JP, Guatelli-Steinberg D, Weston TC, Durner R, Bestsinger TK (2010) Model of tooth morphogenesis predicts Carabelli feature expression, size, and symmetry in humans. *PLoS One* 5, 7 e11844.

Jernvall J (1995) Mammalian molar cusp patterns: Developmental mechanisms of diversity. *Acta Zool Fenn* 198, 1–61.

Jernvall J (2000) Linking development with generation of novelty in mammalian teeth. *Proc Nat Acad Sci U S A* 97, 2641–2645.

Jernvall J, Kettunen P, Karavanova I, Martin LB, Thesleff I (1994) Evidence for the role of the enamel knot as a control center in mammalian tooth cusp formation: non-dividing cells express growth stimulating Fgf-4 gene. *Int J Dev Biol* 38, 463–469.

Jernvall J, Jung HS (2000) Genotype, phenotype, and developmental biology of molar tooth characters. *Year Phys Anthropol* 43, 171–190.

Jernvall J, Thesleff I (2000) Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech Dev* 92, 19–29.

Kimura R, Yamaguchi T, Takeda M, Kondo O, Toma T, Haneji K., et al. (2009) A common variation in EDAR is a genetic determinant of shovel-shaped incisors. *Am J Hum Genet* 85, 528–535.

Klingenberg CP (2002) Morphometrics and the role of the phenotype in studies of the evolution of developmental mechanisms. *Gene* 287, 3–10.

Kollar EJ, Lumsden AG (1979) Tooth morphogenesis: the role of the innervation during induction and pattern formation. *J Biol Buccale* 7, 49–60.

Kondo S, Wakatsuki E, Haung S, Chang S, Sibazaki Y, Arai M (1996) Comparison of the crown dimensions between the maxillary second deciduous molar and the first permanent

molar. *Okajimas Folia Anat Jpn* 73, 179–184.

Korenhof CAW (1960) *Morphogenetical Aspects of the Human Upper Molar*. Utrecht: Uitgeversmaatschappij Neerlandia.

Kraus BS (1952) Morphologic relationships between enamel and dentin surfaces of lower first molar teeth. *J Dent Res* 31, 248–256.

Kraus BS, Jordan RE (1965) *The Human Dentition Before Birth*. Philadelphia: Lea and Febiger.

Lumsden AG (1987) The neural crest contribution to tooth development in the mammalian embryo. In: *Developmental and Evolutionary Aspects of the Neural Crest* (ed. Maderson FP), pp. 261–300. New York: John Wiley.

Lumsden AG (1988) Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. *Development* 103(Suppl), 155–169.

Mitsidis TA, Smith MM (2006) How do genes make teeth to order through development? *J Exp Zool* 306B, 177–182.

Nager G (1960) Der vergleich zwischen dem räumlichen verhalten des dentinkronenreliefs und dem schmelzrelief der zahnkrone. *Acta Anat* 42, 226–250.

Olejniczak AJ, Gilbert CG, Martin LB, Smith TM, Ulhaas L, Grine FE (2007) Morphology of the enamel-dentine junction in sections of anthropoid primate maxillary molars. *J Hum Evol* 53, 292–301.

Ortiz A, Skinner MM, Bailey SE, Hublin JJ (2012) Carabelli's trait revisited: An examination of mesiolingual features at the enamel-dentine junction and enamel surface of Pan and Homo sapiens upper molars. *J Hum Evol* 63, 586–596.

Pilbrow V (2006) Population systematics of chimpanzees using molar morphometrics. *J Hum Evol* 51, 646–662.

Sakai T, Hanamura H (1971) A morphological study of enamel-dentin border on the Japanese dentition: V. Maxillary molar. *J Anthropol Soc Nippon* 79, 297–322.

Sakai T, Hanamura H (1973) A morphological study of enamel-dentin border on the Japanese dentition: VII. General conclusion. *J Anthropol Soc Nippon* 81, 87–102.

Salazar-Ciudad I, Jernvall J (2002) A gene network model accounting for development and

evolution of mammalian teeth. *Proc Natl Acad Sci U S A*. 99, 8116–8120.

Sasaki K, Kanazawa E (1999) Morphological traits on the dentino-enamel junction of lower deciduous molar series. In: *Dental morphology 1998: Proceedings of the 11th international symposium on dental morphology* (eds. Mayhall J, Heikkinen T), pp. 167–178. Oulu: Oulu University Press.

Schwartz GT (2000) Taxonomic and functional aspects of the patterning of enamel thickness distribution in extant large-bodied hominoids. *Am J Phys Anthropol* 111, 221–244.

Scott GR, Turner II CG (1997) *The Anthropology of Modern Human Teeth*. Cambridge: Cambridge University Press.

Sharpe PT (1995) Homeobox genes and orofacial development. *Connect Tissue Res* 32, 17–25.

Skinner MM, Gunz P, Wood BA, Hublin JJ (2008a) Enamel-dentine junction (EDJ) morphology distinguishes the lower molars of *Australopithecus africanus* and *Paranthropus robustus*. *J Hum Evol* 55, 979–988.

Skinner MM, Wood BA, Boesch C, Olejniczak AJ, Rosas A, Smith TM, Hublin JJ (2008b) Dental trait expression at the enamel-dentine junction of lower molars in extant and fossil hominoids. *J Hum Evol* 54, 173–186.

Skinner MM, Wood BA, Hublin JJ (2009) Protostylid expression at the enamel-dentine junction and enamel surface of mandibular molars of *Paranthropus robustus* and *Australopithecus africanus*. *J Hum Evol* 56, 76–85.

Skinner MM, Evans A, Smith T, et al. (2010) Brief Communication: Contributions of enamel-dentine junction shape and enamel deposition to primate molar crown complexity. *Am J Phys Anthropol* 142, 157–163.

Smith TM, Olejniczak AJ, Reid DJ, Ferrell R, Hublin JJ (2006) Modern human molar enamel thickness and enamel dentine junction shape. *Arch Oral Biol* 51, 974–995.

Smith P, Muller R, Gabet Y, Avishai G (2007) A computerized model for reconstruction of dental ontogeny: A new tool for studying evolutionary trends in the dentition. In: *Dental perspectives on human evolution* (eds. Bailey SE, Hublin JJ), pp. 273–286. AA Dordrecht, The Netherlands: Springer.

- Smith P, Avishai G, Muller R, Gabet Y (2011) Computerized reconstruction of prenatal growth trajectories in the dentition: Implications for the taxonomic status of Neandertals. In: *Continuity and discontinuity in the peopling of Europe: One hundred fifty years of Neanderthal study* (eds. Condemi, Weniger GC), pp. 165–173. New York: Springer Science+Business Media B.V.
- Suwa G, Wood BA, White TD (1994) Further analysis of mandibular molar crown and cusp areas in Pliocene and Early Pleistocene hominids. *Am J Phys Anthropol* 93, 407–426.
- Suwa G, White TD, Howell FC (1996) Mandibular postcanine dentition from the Shungura Formation, Ethiopia: crown morphology, taxonomic allocations, and Plio-Pleistocene hominid evolution. *Am J Phys Anthropol* 101, 247–282.
- Suwa G, Kono RT, Katoh S, Asfaw B, Beyene Y (2007) A new species of great ape from the late Miocene epoch in Ethiopia. *Nature* 448, 921–924.
- Tafforeau P, Bentaleb I, Jaeger JJ, Martin C (2007) Nature of laminations and mineralization in rhinoceros enamel using histology and X-ray synchrotron microtomography: potential implications for palaeoenvironmental isotopic studies. *Palaeogeogr Palaeoclimatol Palaeoecol* 246, 206–227.
- TenCate AR (2012) Oral Histology: Development, Structure and Function, 8th Edition. St. Louis: C.V. Mosby Company.
- Thesleff I (2003) Epithelial-mesenchymal signalling regulating tooth morphogenesis. *J Cell Sci* 116, 1647–1648.
- Tummers M, Thesleff I (2009) The importance of signal pathway modulation in all aspects of tooth development. *J Exp Zool B Mol Dev Evol* 312B, 309–319.
- Vahtokari A, Aberg T, Jernvall J, Keränen S, Thesleff I (1996) The enamel knot as a signaling center in the developing mouse tooth. *Mech Dev* 54, 39–43.
- Wagner GP, Altenberg L (1996) Complex adaptations and the evolution of evolvability. *Evolution* 50, 967–976.
- Wagner GP, Booth G, Bagheri-Chaichian H (1997) A population genetic theory of canalization. *Evolution* 51, 329–347.
- Willmore KE, Young N, Richtsmeier JT (2007) Phenotypic variability: its components,

measurement and underlying developmental processes. *Evol Biol* 34, 99–120.

Yamada H, Brown T (1988) Contours of maxillary molars studied in Australian Aborigines. *Am J Phys Anthropol* 76, 399–407.

Zhang YD, Chen Z, Song YQ, Liu C, Chen YP (2005) Making a tooth: growth factors, transcription factors and stem cells. *Cell Res* 15, 301–316.

Chapter 2: Size and shape variability in human molars during odontogenesis

Abstract

Under the patterning cascade model (PCM) of cusp development inspired by developmental genetic studies, it is predicted that the location and the size of later-forming cusps are more variable than those of earlier-forming ones. Here we assessed whether differences in the variability among cusps at total and each particular crown components (enamel-dentin junction [EDJ], outer enamel surface [OES], and cement-enamel junction [CEJ]) could be explained by the PCM, using human maxillary permanent first molars (UM1) and second deciduous molars (um2). Specimens were μ CT-scanned, and 3D models of EDJ and OES were reconstructed. Based on these models, landmark-based 3D geometric morphometric analyses were conducted. Size variability in both tooth types was generally consistent with the above prediction, and the differences in size variation among cusps were smaller for the crown components that are completed in later stages of odontogenesis. With a few exceptions, however, the prediction was unsupported regarding shape variability, and UM1 and um2 showed different patterns. Our findings suggested that the pattern of size variability would be caused by temporal factors such as the order of cusp initiation and the duration from the beginning of mineralization to the completion of crown formation, whereas shape variability may be affected by both topographic and temporal factors.

Key Words: dental morphology, morphometrics, morphogenesis, dental anatomy

Introduction

In multicuspidate teeth, secondary enamel knots appear sequentially at the future location of each cusp and repeatedly use the same signaling pathways (Jernvall and Jung, 2000). The spatial patterning and number of cusps are determined by the iterative activation of secondary enamel knots and by reciprocal signaling within and between oral epithelium and mesenchyme (Patterning Cascade Model: PCM; Jernvall, 2000). In this model, cusp initiation is sequential, and the location and size of later-forming cusps are influenced by those of earlier-forming ones (Salazar-Ciudad *et al.*, 2003).

If the positioning of later-forming secondary enamel knots is dependent on the positioning of the pre-existing secondary enamel knots and if the perturbations in earlier cascade events are amplified in later events, it is very likely that the variation of the morphology of later-forming cusps will surpass that of early-forming cusps (Jernvall, 1997; Polly, 1998). This prediction was supported concerning the cusp height and position in seal dentition (Jernvall, 1997, 2000), and also received support from studies of cusp size variability (Townsend *et al.*, 2003; Harris and Dihn, 2006; Takahashi *et al.*, 2007) and Carabelli cusp expression (Hunter *et al.*, 2010) in the human molars. However, Polly (1998) found that earlier-forming cusps were more variable in their positions than later-forming cusps in viverravid molar, and proposed that the order of cusp initiation and the timing of the termination of intercusp growth determine patterns of variability in cusp position and height. Polly (1998) mentioned the possibility that initial difference in variability among cusps might be obliterated in human molar that had a long gap between cusp initiation and the termination of intercusp growth because developmental perturbations could have a cumulatively greater effect on earlier-forming cusps.

To understand the precise variability-generating mechanisms regulated by the PCM, it is necessary to obtain detailed information about differences of morphological variability among cusps, about which there remains a dearth of information. For example, except for the spatial distribution of cusp tips in mammalian molars (Jernvall, 1997, 2000; Polly, 1998), little attention has been paid to cusp *shape* variability. Previous studies have principally focused on the outer enamel surface (OES) morphology of the occlusal surface (Corruccini, 1979;

Harris and Dihn, 2006; Takahashi *et al.*, 2007). The results obtained could be explained by the PCM that morphological variability becomes larger in the later-forming cusps. However, the PCM would relate more directly to cusp patterning at EDJ (Skinner and Gunz, 2010) than to the other parts of crown components – OES-ridge, OES-circumferences, and cement-enamel junction (CEJ) – that are elaborated through the subsequent developmental processes, including enamel matrix deposition and the elongation of cervical loop (Butler, 1956; Jernvall and Jung, 2000). Could differences in the variability among cusps at total and each particular crown components be explained by the PCM? Comparing morphological variability among cusps at these components could provide significant information about the variability-generating mechanisms during odontogenesis, which would be relevant to morphological evolution because developmental process structures morphological variation on which natural selection can act, which biases the developmental processes available for subsequent generations.

Here, we examined the pattern of morphological variability among cusps of the maxillary permanent first molar (UM1) and second deciduous molar (um2). They have similar main-cusp and occlusal groove patterns and belong to the same molarization field (Butler, 1967), which does not contradict recent findings of molecular, cellular or genetic studies (Sharpe, 1995; Mitsidis and Smith, 2006). Although UM1 and um2 share similar patterns of occlusal morphology, UM1 is larger than um2 in size. Additionally, um2 crown is initiated 12.5-19 weeks after fertilization and is completed by 11 months after birth, whereas UM1 crown begins to calcify at birth and is completed at 2.6-2.7 years (TenCate, 2012). Thus, developmental timing, period, and rate are distinct between UM1 and um2, which enables us to explore their effects on patterns of morphological variability. Specifically, we tested the following hypotheses.

Hypothesis 1: later-forming cusps have greater *size variability* than earlier-forming ones, and this holds for each crown component (EDJ-ridge, OES-ridge, OES-circumferences, and CEJ).

Hypothesis 2: the *shape variability* of later-forming cusps is greater than that of earlier-forming ones, and this holds for each crown component.

Hypothesis 3: UM1 and um2 share common patterns of size and shape variability for each crown component.

Materials and Methods

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

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

We digitized each main cusp (paracone, protocone, metacone, and hypocone) region of four crown components (EDJ-ridge, OES-ridge, OES-circumferences, and CEJ) in a tooth (more details in Supplementary data in this chapter). The dataset was represented by four coordinate matrices comprising a total of 8 landmarks and 84 semi-landmarks (Figure 1A-B).

Centroid size (CS) was calculated in particular components of cusps. Coefficient

of variation (CV) of the CS was used to compare size variability, and tested as suggested by Sokal and Braumann (1980).

For comparison of shape variability among cusps, Generalized Procrustes Analysis (GPA; Rohlf and Slice, 1990) was performed using MorphoJ version 1.05d (Klingenberg, 2011). To include the information of relative spatial distribution among cusps, GPA was repeated for the landmark set of the total and each crown component. 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 variability (Polly, 1998; Jernvall, 2000). To test whether there was a significant difference in variation among cusps, a nonparametric Kruskal-Wallis test and multiple-comparison test were performed. The correlation between the shape variability and the order of cusp initiation (paracone, protocone, metacone, and hypocone: Turner, 1963; Kraus and Jordan, 1965) was assessed using Spearman's rank coefficient. All statistical analyses were performed using R version 2.13.1 (R Development Core Team, 2011), with statistical significance set at $P < 0.05$.

Results

Hypothesis 1 (greater size variability in later-forming cusps).

The CV of total crown components of UM1 did not show any significant difference, although the last-forming hypocone had a somewhat greater variation (Figure 1C). For EDJ-ridge, the hypocone had a larger CV than the other cusps ($P=0.074$). For OES-ridge, the hypocone showed higher variability than the other cusps, but the difference in variability was not significant ($P=0.397$). For OES-circumferences, earlier-forming cusps showed slightly higher variability, but the difference among cusps was not significant ($P=0.895$). For CEJ, the later-forming metacone was more variable, but there was not significant difference among cusps ($P=0.430$). In summary, hypothesis 1 was unsupported in UM1, but there was a tendency of higher size variability in later-forming cusps for EDJ-ridge.

The difference in size variation among cusps was pronounced in um2 (Figure 1D). For every topological feature except CEJ, the hypocone showed significantly higher variability than other cusps. In the case of CEJ, although the hypocone tended to be more variable, no

significant difference was observed ($P=0.169$), and the difference among cusps was smaller than that for other parts of the crown components.

Hypothesis 2 (greater shape variability in later-forming cusps).

In UM1, a negative correlation was observed between shape variability and the cusp initiation order for OES-circumferences ($P<0.001$) and CEJ ($P=0.026$) (Figure 2). No correlation existed for other components. In the case of OES-circumferences, a nonparametric multiple-comparison test showed that the hypocone was significantly less variable in shape than the paracone ($P=0.028$) and protocone ($P=0.030$). In the case of CEJ, the metacone was less variable than the paracone ($P=0.014$). These results did not support hypothesis 2, and were also inconsistent with the order of cusp initiation.

In um2, a positive correlation was observed between shape variability and the cuspal initiation order for total crown components ($P=0.010$) and OES-ridge ($P=0.004$) (Figure 3). Direct comparisons of total crown components revealed that the hypocone was more variable than the paracone ($P=0.018$). Moreover, for OES-ridge, Kruskal-Wallis analysis revealed significant difference among cusps ($P=0.031$), and the paracone was less variable than the hypocone ($P=0.048$). For CEJ, there was significant difference among cusps ($P=0.016$), and the metacone was less variable than the protocone ($P=0.058$), although the correlation with the order of cusp initiation was not significant ($P=0.554$). As a whole, lingual cusps (protocone and hypocone) were more variable than buccal cusps (paracone and metacone). Therefore, hypothesis 2 was supported only for total crown components and OES-ridge.

Hypothesis 3 (UM1 and um2 share common patterns of size and shape variability).

As noted above, the tendency of greater size variability of later-forming cusps at least in EDJ was common between UM1 and um2, whereas the shape variability showed a tooth-specific pattern. Then, hypothesis 3 was supported only partially for size variability, and was refuted for shape variability.

Discussion

Human molars grow substantially after the cusps form, and then this growth might hide any small differences in cusp height and size (Butler, 1956). However, recent developmental analysis revealed that crown sizes were regulated by intrinsic factors from mesenchymal tissues (Cai *et al.*, 2007). Because the secondary enamel knots are induced in a sequential cascade, when a broader inhibition field which is controlled by the nested expression and interaction of activator and inhibitor proteins (Jernvall and Jung, 2000) around earlier-forming enamel knot increases cusp spacing, later-forming cusps will be smaller and *vice versa*. Therefore, it is expected that the size of earlier-forming cusps will be larger at the expense of the later-forming cusps if the rate of formation of the earlier-forming cusps is faster and/or the duration of their formation is longer than those of the later-forming cusps (Takahashi *et al.*, 2007). This causes relatively larger size variability in later-forming cusps, which can be observed in not only OES but also various parts of the crown components albeit mineralization process does not interact across cusps.

Temporal factors during odontogenesis, such as the order of cusp initiation and the duration from the beginning of mineralization to the completion of crown formation, are likely responsible for the pattern of size variability. The differences in size variation among cusps are greater in the earlier-forming crown components (in particular EDJ-ridge) and the influence of the order of cusp initiation is smaller in the later-forming components. UM1 and um2 differ regarding how much and how long the later-forming cusps are susceptible to variability in size. The difference in variability among cusps in um2 is greater than that in UM1, and it is preserved in the later phase of development. Because the developmental period of UM1 is longer than that of um2 (Liversidge and Molleson, 2004), the relatively large size variability of earlier-forming cusps in UM1 probably results from greater cumulative perturbation over a longer period of odontogenesis, whereas the relatively shorter developmental period of um2 leads to the lasting effect of the order of cusp initiation. Polly (1998) stressed that initial differences in height and variability among cusps might be erased when there was a long delay between the enamel knot activation and the intercusp growth termination. The present study suggests that this idea may be applicable to the whole process of the odontogenesis. Apart from temporal factors for size variability, natural selection on occlusion can cause

smaller variability of earlier-forming cusps consisted of the trigon, which might explain clearer tendency at EDJ and OES which are more responsible for occlusion and in um2 which preserves primitive morphology (Butler, 1956).

Unlike size variability, shape variability in UM1 did not show patterns consistent with the PCM-based hypothesis of greater variability in later-forming cusps. Rather, the earlier-forming cusps were more variable than the later-forming cusps regarding OES-circumferences and CEJ, which could be explained by applying Polly's (1998) previously mentioned idea. The greater variability of the earlier-forming cusps reflects a greater effect of cumulative perturbation due to the longer period of development. However, there was no significant difference between the later-forming and earlier-forming cusps regarding variability in shape during the earlier stage of odontogenesis. This might be the result of complicated effects of the order of cusp initiation, cumulative perturbations of the longer developmental period, and/or unknown developmental factors.

The patterns of shape variability of um2 were consistent with the order of cusp initiation for OES-ridge, but not for EDJ-ridge. In the case of EDJ-ridge, the hypothesized pattern might have been erased by multifactorial effects during development. The pattern of shape variability of the later-forming OES-ridge might result from the order of cusp initiation amplified by enamel deposition. In the later stage of odontogenesis, the shape of lingual cusps is more variable than that of buccal ones. This may be explained by several developmental factors, such as the lingual side-dominated growth pattern, the spatial relationship with the surrounding tissues including maxillary bone and/or other tooth germs, and the available space for tooth growth (Boughner, 2011), which might have more influence on the patterns of shape variability, than the effect of cumulative perturbation due to the longer period of development.

The size variability of human molar cusps follows the theoretical explanations proposed by Jernvall (2000). However, with a few exceptions, the hypothesized variability pattern was not observed regarding cusp shape variability, and instead, UM1 and um2 showed different patterns of shape variability from each other. During odontogenesis, temporal factors would contribute to the patterns of size variability, whereas shape variability might be more influenced by topological factors.

References

- Boughner (2011) Making space for permanent molars in growing baboon (*Papio anubis*) and great ape (*Pan paniscus* and *P. troglodytes*) mandibles: Possible ontogenetic strategies and solutions. *Anat Res Int* <http://dx.doi.org/10.1155/2011/484607>.
- Butler PM (1956) The ontogeny of molar pattern. *Biol Rev* 31, 30–70.
- Butler PM (1967) Comparison of the development of the second deciduous molar and first permanent molar in man. *Arch Oral Biol* 12, 1245–1260.
- Cai J, Cho SW, Kim JY, Lee MJ, Cha YG, Jung HS (2007) Patterning the size and number of tooth and its cusps. *Dev Biol* 304, 499–507.
- Corruccini RS (1979) Molar cusp-size variability in relation to odontogenesis in hominoid primates. *Arch Oral Biol* 24, 633–634.
- Harris EF, Dinh DP (2006) Intercusp relationships of the permanent maxillary first and second molars in American whites. *Am J Phys Anthropol* 130, 514–528.
- Hunter JP, Guatelli-Steinberg D, Weston TC, Durner R, Betsinger TK (2010) Model of Tooth Morphogenesis Predicts Carabelli Cusp Expression, Size, and Symmetry in Humans. *Plos ONE* 5 e11844. doi:10.1371/journal.pone.0011844.
- Jernvall J (1995) Mammalian molar cusp patterns: Developmental mechanisms of diversity. *Acta Zoologica Fennica* 198, 1–61.
- Jernvall J (1997) Developmental basis of mammalian molar cusp evolution. *J Vert Paleontol* 17(Suppl), 56A.
- Jernvall J (2000) Linking development with generation of novelty in mammalian teeth. *Proc Nat Acad Sci USA* 97, 2641–2645.
- Jernvall J, Jung HS (2000) Genotype, phenotype, and developmental biology of molar tooth characters. *Year Phys Anthropol* 43, 171–190.
- Klingenberg CP (2011) MorphoJ: an integrated software package for geometric morphometrics. *Molec Ecol Res* 11, 353–357.
- Kraus BS, Jordan RE (1965) The Human Dentition Before Birth. Philadelphia: Lea and Febiger.

- Liversidge HM, Molleson T (2004) Variation in crown and root formation and eruption of human deciduous teeth. *Am J Phys Anthropol* 123, 172–180.
- Mitsidis TA, Smith MM (2006) How do genes make teeth to order through development? *J Exp Zool* 306B, 177–182.
- Polly PD (1998) Variability, selection, and constraints: development and evolution in viverravid (Carnivora, Mammalia) molar morphology. *Paleobiology* 24, 409–429.
- R Development Core Team (2011) R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. <http://cran.R-project.org>.
- Rohlf FJ, Slice D (1990) Extensions of the Procrustes method for the optimal superimposition of landmarks. *Syst Zool* 39, 40–59.
- Salazar-Ciudad I, Jernvall J, Newman, SA (2003) Mechanisms of pattern formation in development and evolution. *Development* 130, 2027–2037.
- Skinner MM, Gunz P (2010) The presence of accessory cusps in chimpanzee lower molars is consistent with a patterning cascade model of development. *J Anat* 217, 245–253.
- Sharpe PT (1995) Homeobox genes and orofacial development. *Connect Tissue Res* 32, 17–25.
- Sokal RR, Braumann CA (1980). Significance tests for coefficients of variation and variability profiles. *Syst Zool* 29, 50–66.
- Takahashi M, Kondo S, Townsend GC, Kanazawa E (2007) Variability in cusp size of human maxillary molars, with particular reference to the hypocone. *Arch Oral Biol* 52, 1146–54.
- TenCate AR (2012) Oral Histology: Development, Structure and Function, 8th Edition. St. Louis: C.V. Mosby Company.
- Townsend G, Richards L, Hughes T (2003) Molar intercuspal dimensions: genetic input to phenotypic variation. *J Dent Res* 82, 350–355.
- Turner EP (1963) Crown development in human deciduous molar teeth. *Arch Oral Biol* 8, 523–550.

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Supplementary Data

The cervical line of each tooth was manually traced using an interpolation curve tool to generate the best-fit plane. The tooth was then aligned so that this plane fit the xy -plane of the Cartesian coordinate system where the centroid of the cervical line defined the origin.

OES-ridge that connects adjacent cusp tips is separated at the lowest points (breakpoints) on which are located between the two cusps. The cusp tips and breakpoints divide the whole OES-ridge into eight sections. For each section, two semi-landmarks were set so that the ridge length is divided into equal one-thirds.

OES-circumferences (outlines) are traced at one-thirds and two-thirds of the height between the protocone tip and the cervical plane. Each circumference is divided into four sections (corresponding to the four cusp regions) by the inter-cuspal grooves. Ten semi-landmarks (the sum of five semi-landmarks of each circumference) are digitized in each section equi-angularly at the intersection of each OES-circumference with a plane perpendicular to the xy -plane passing through the origin.

The same procedure as used for the OES-ridge was performed on the dentin horn and EDJ-ridge, and the ridge length of each section is divided at the midpoint by one semi-landmark.

CEJ curve is also traced and divided into four sections at the most internally protuberant points between the adjacent two cusp regions. For each section, 5 semi-landmarks that divide the section into 6 parts equi-distantly are taken.

The number of semi-landmarks on the EDJ and the OES were determined to satisfy two criteria: 1) that each cusp has the same number of (semi)landmarks and 2) that the contributions of sections between (semi)landmarks to the curve are relatively equal to each other (Skinner et al., 2009; Skinner and Gunz, 2010).

Each four crown component (EDJ-ridge, OES-ridge, OES-circumferences, CEJ) is divided into 4 cusp regions that span from breakpoint to breakpoint. Those breakpoints are not included in the subsequent analyses. Finally, the dataset was represented by four coordinate matrices comprising a total of 8 landmarks and 84 semi-landmarks (Supplementary Figure 1: 2 landmarks and 21 semi-landmarks for each of the four cusps).

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).

Centroid size (CS), defined as the square root of the summed squared distances of the coordinates from their centroid, of each cusp in the total and each crown component was calculated. Coefficient of variation (CV) of the CS of each cusp was used as a measure of size (not height) variability. For comparisons of shape variability among cusps, Generalized Procrustes Analysis (GPA) was repeated for the landmark set of the total and each crown component in order to include the information of relative spatial distribution among cusps. 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 variability.

References

- Bookstein FL (1997) Landmark methods for forms without landmarks: morphometrics of group differences in outline shape. *Med Image Anal* 1, 225–243.
- Gunz P, Mitteroecker P, Bookstein FL (2005) Semilandmarks in three dimensions. In: *Modern Morphometrics in Physical Anthropology* (ed. Slice DE), pp. 73–98. New York: Kluwer Academic/Plenum Publishers.
- Skinner MM, Gunz P, Wood BA, Boesch C, Hublin J (2009) Discrimination of extant *Pan* species and subspecies using the enamel-dentine junction morphology of lower molars. *Am J Phys Anthropol* 140, 234–243.
- Skinner MM, Gunz P (2010) The presence of accessory cusps in chimpanzee lower molars is

consistent with a patterning cascade model of development. *J Anat* 217, 245–253.

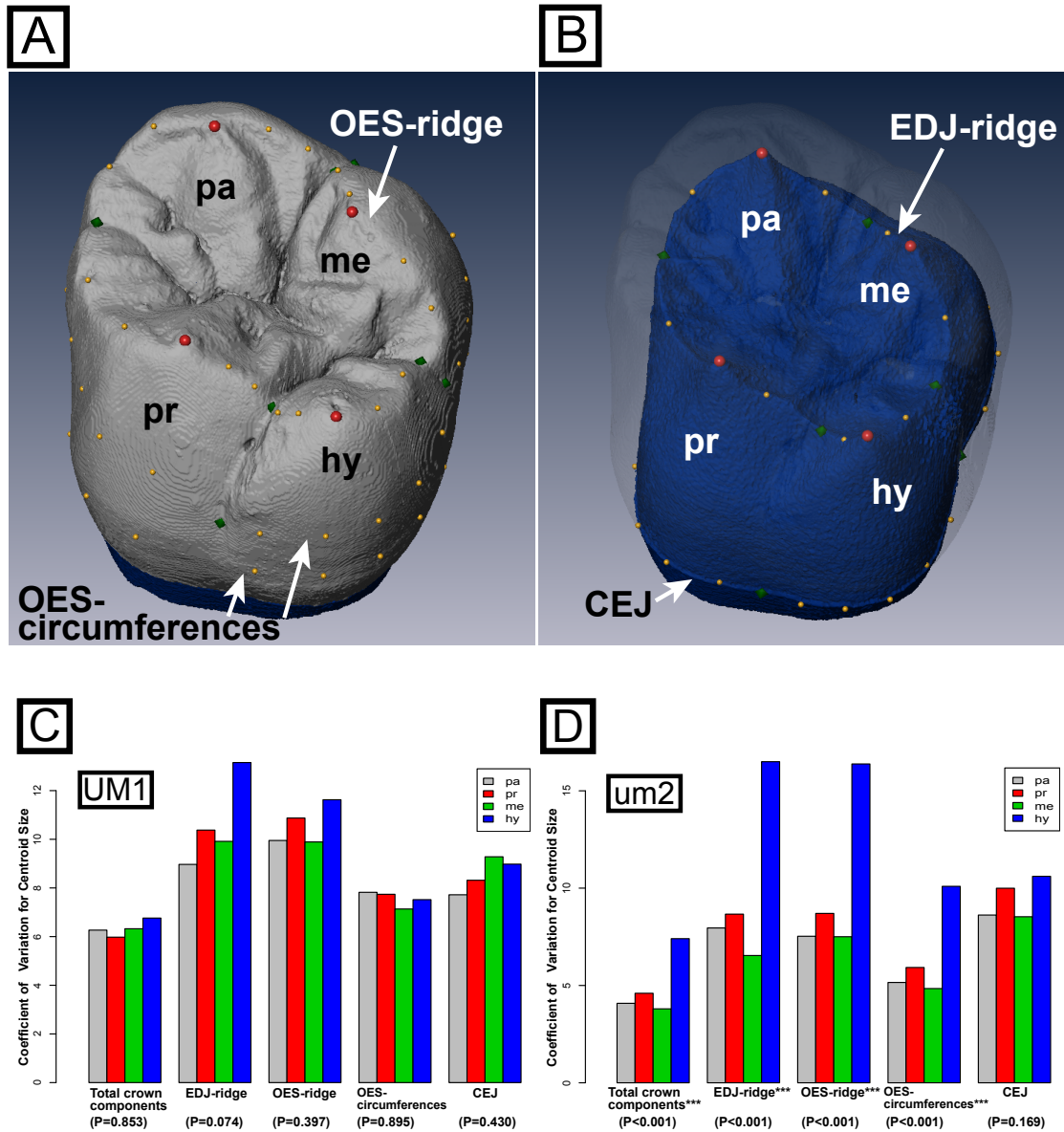


Figure 1. Digital image of maxillary permanent first molar crown (lingual view) and barplot of patterns of size variability. (A) OES-ridge curve and OES-circumferences digitized on the OES. (B) EDJ-ridge curve and CEJ curve digitized on the EDJ surface. Red circles are landmarks, yellow circles are semi-landmarks, and green rhomboids are breakpoints. pa, paracone; pr, protocone; me, metacone; hy, hypocone. (C) Barplot of patterns of size variability in UM1. (D) Barplot of patterns of size variability in um2. Significance tests for coefficients of variation for centroid size among cusps were performed following the recommendations of Sokal and Braumann (1980). There was a tendency of higher size variability in later-forming cusps, and the variability difference among cusps was smaller in the later-forming components.

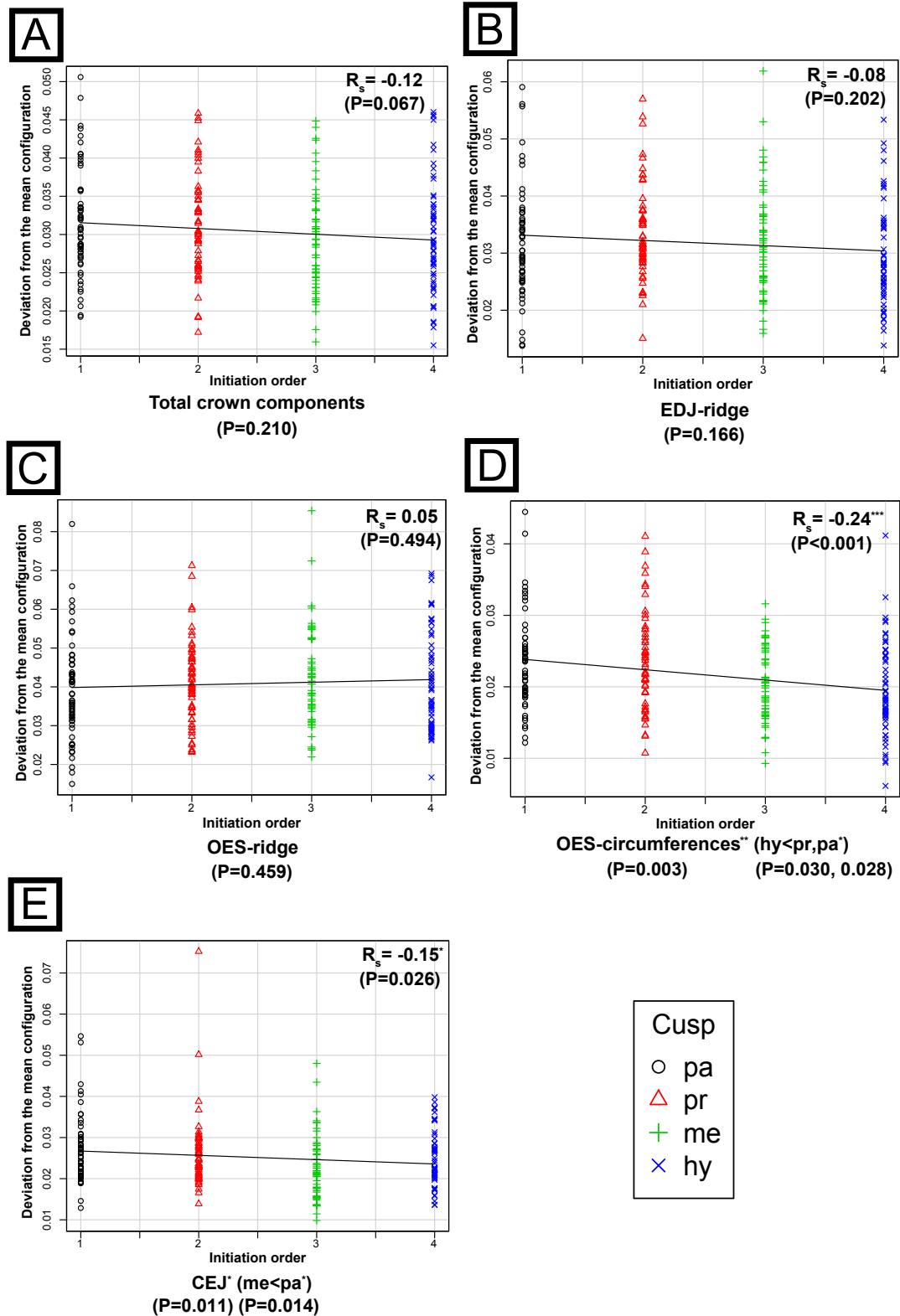


Figure 2. Patterns of shape variability in UM1. Relationship between variability and cusp initiation order is shown for total crown components (A), EDJ-ridge (B), OES-ridge (C), OES-circumferences (D), and CEJ (E). Differences among cusps were tested by Kruskal-Wallis test, followed by nonparametric multiple-comparison test. R_s , Spearman's rank correlation coefficients; * $P<0.05$; ** $P<0.01$; *** $P<0.001$. The greater variability of the earlier-forming cusps was observed in the later-forming

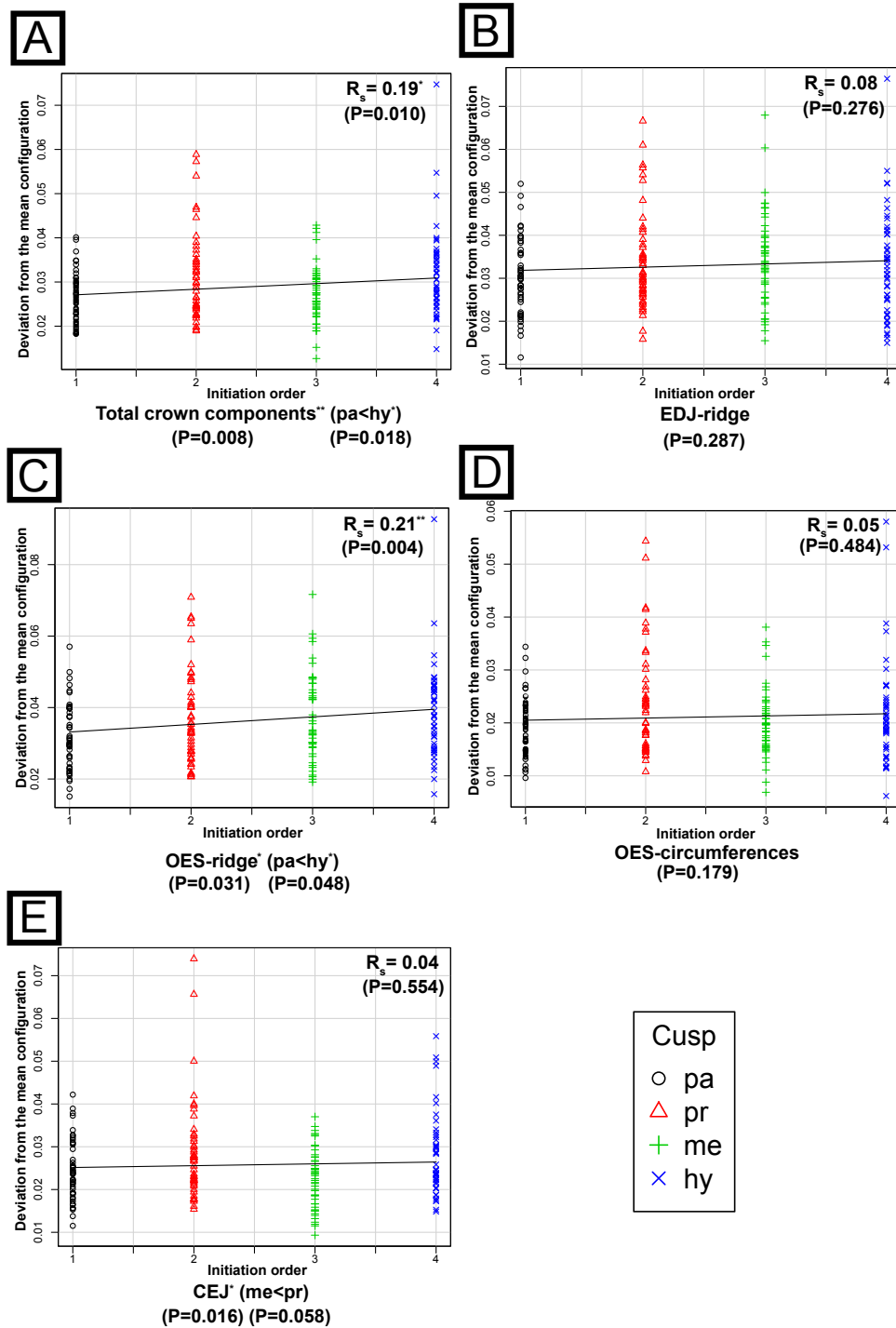
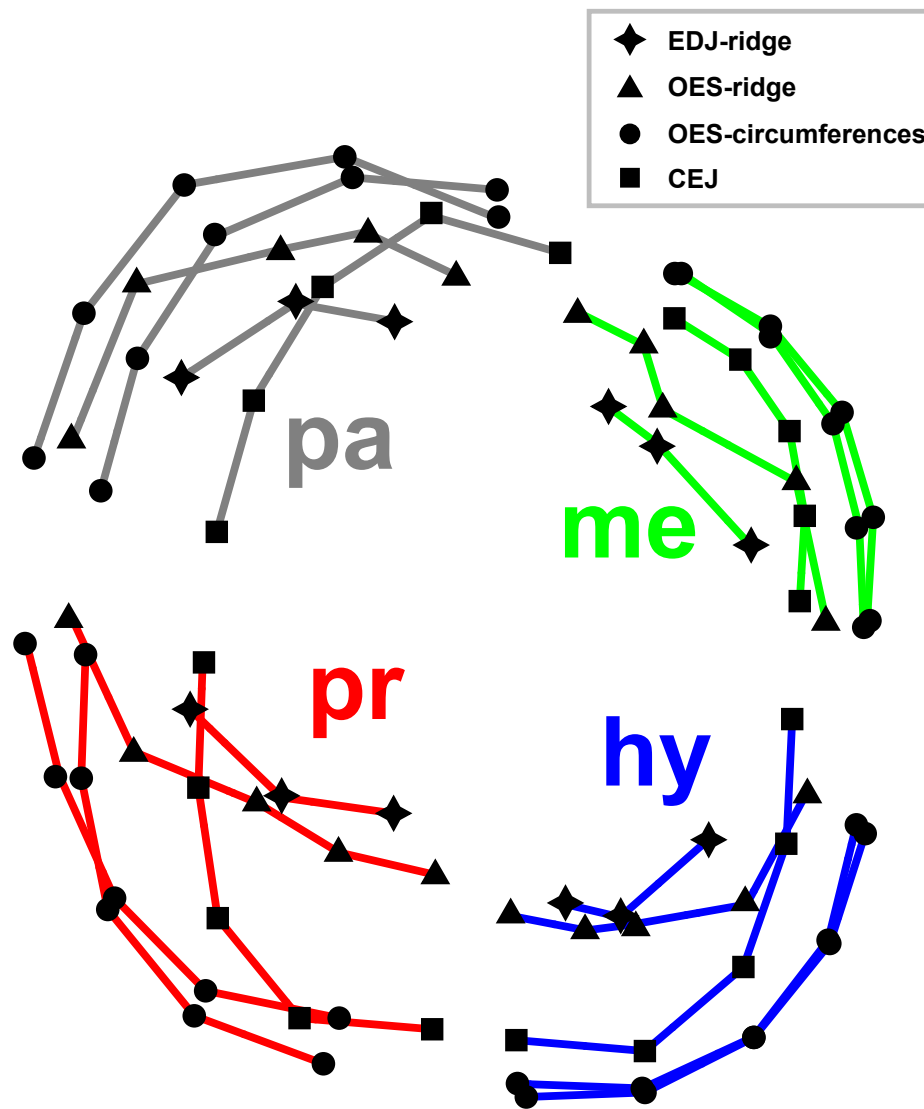


Figure 3. Patterns of shape variability in um2. Relationship between variability and cusp initiation order is shown for total crown components (A), EDJ-ridge (B), OES-ridge (C), OES-circumferences (D), and CEJ (E). Differences among cusps were tested by Kruskal-Wallis test, followed by nonparametric multiple-comparison test. R_s , Spearman's rank correlation coefficients; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Later-forming cusps showed greater variability in total crown component and OES-ridge whereas lingual cusps showed greater variability in CEJ.



Supplementary Figure 1. Each four cusp delineation of maxillary permanent first molar crown (lingual view) is represented by landmarks connecting each other on the same crown component in a cusp. Landmarks, including semi-landmarks after slid, are represented by stars: on EDJ-ridge, triangles: on OES-ridge, circles: on OES-circumferences, squares: on CEJ. EDJ-ridge and OES-ridge are divided into four cusp regions by break points that are located at the lowest points between adjacent two dentin horns or cusp tips. Two OES-circumferences are traced at one-thirds and two-thirds of the height between the protocone tip and the cervical plane and divided into four sections, corresponding to the four cusp regions, by the inter-cuspal grooves. CEJ curve is divided into four sections at the most internally protuberant points between the adjacent two cusp regions. Each cusp has a total of 23 landmarks (2 landmarks and 21 semi-landmarks). pa, paracone; pr, protocone; me, metacone; hy, hypocone.

Chapter 3: Canalization, morphological integration, and evolutionary flexibility in enamel-dentin junction and outer-enamel surface of human molars

Abstract

Phenotypic variability is the tendency or potential of an organism to vary and determines the potential range of morphological variation. This study compared the phenotypic variability of enamel-dentin junction (EDJ) and outer-enamel surface (OES) in human maxillary permanent first molar (UM1) and second deciduous molar (um2). Using geometric morphometric methods, we evaluated the strength of two components of phenotypic variability: canalization and morphological integration, plus the relevant evolutionary flexibility, i.e., the ability to respond to selective pressure. The lack of a significant difference in size variation between EDJ and OES suggested that the strength of canalization on size was almost constant throughout odontogenesis. In UM1, EDJ showed less shape variation and a higher level of morphological integration than OES, which indicated that canalization and morphological integration acted as developmental constraints. In um2, such a tendency appeared weaker, probably due to the thinner enamel and/or shorter period of enamel formation. Evolutionary flexibility was not significantly different between EDJ and OES in either tooth, possibly because the pattern cascade mode of cusp development would cause a cusp-divided covariance structure. This crown formation would lead to the retention of a certain level of evolvability of EDJ despite the existence of developmental constraints.

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

Introduction

Phenotypic variability is defined as the tendency or potential of an organism to vary (Wagner and Altenberg, 1996; Wagner et al., 1997; Willmore et al., 2007). Therefore, it determines the potential range or distribution of morphological variation, and ultimately affects the tempo and mode of evolutionary change. The recent literature about phenotypic variability has paid the greatest attention to canalization and morphological integration (Wagner and Altenberg, 1996; Hallgrímsson et al., 2002; Willmore et al., 2007; Hallgrímsson et al., 2009). Canalization is generally considered a property of an organism that limits 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).

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 form 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. Ameloblasts are derived from the IEE cells and odontoblasts from cells of the dental papilla. 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 to dental functions such as occlusion and feeding and thus is a direct target of natural selection, the morphology of EDJ has been considered to be more conservative evolutionally and a more reliable representation of the phenotype for estimating phylogenetic relationships (Kraus, 1952; Korenhof, 1960; Smith et al., 1997; Sasaki and Kanazawa, 1999; Smith et al., 2000; Olejniczak et al., 2007). As such, given the different developmental timing between the EDJ and OES, it is likely that the patterns of phenotypic variability differ between these structures. Comparison of the pattern of variability between EDJ and OES should provide a clue about how the differences in their developmental processes bias the production of morphological variation and would influence inferred phylogenetic reconstructions.

For this reason, here we specifically test the following three null hypotheses between the EDJ and the OES morphological variability in a single tooth crown.

Hypothesis 1: *no difference in canalization of size or shape*. If the null hypothesis

is rejected and 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.

Hypothesis 2: *no difference in morphological integration*. If the null hypothesis is rejected, it means that, either during morphogenesis or the enamel formation process, some developmental factors produce higher morphological integration of one of these structures (whether EDJ or OES). And combined with the result regarding hypothesis 1, this analysis will help to determine what factors play important roles in generating or reducing morphological variance.

Hypothesis 3: *no difference in evolutionary flexibility*. In relation to the above 2 hypotheses, we specifically compared how the developmental constraints exert influence on the ability of the response to selection in EDJ versus in OES. In addition to the above hypotheses, we tested another null hypothesis:

Hypothesis 4: *UM1 and um2 share common patterns of canalization, morphological integration, and evolutionary flexibility in the EDJ and the OES*. 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 (Ten Cate, 2012). If the null hypothesis is rejected, these differences are assumed to affect the patterns of morphological variability. Comparing the patterns of variability between UM1 and um2 will provide a better understanding of the relationship between odontogenesis and crown morphological variability.

Materials and Methods

The samples used in this study comprised fully formed but unworn UM1 and um2 crowns obtained from archaeological sites in Japan. The total sample (57 UM1 and 48 um2) consisted of samples from the Jomon (14500-300 BC; n=8 and 5), Medieval (13-15C AD;

n=13 and 8), and Edo (17-19C AD; n=36 and 35) periods (Appendix). Although the total sample was from a mixture of populations from different periods and regions, the aim of this study was to investigate differences and patterns of variability produced by a common tooth formation process of the Holocene human, and mixing these samples does not violate the objective of this study. In order to maximize sample size, no discrimination between right and left teeth was made, but only a single tooth was used from each individual. All specimens were regarded as left side. Right molar images were transformed into the mirror image using ImageJ software (NIH, USA). Sex was unknown for most of the samples, since they were taken from juvenile individuals.

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

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 Kruskal-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 the OES. 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

Hypothesis 1: no difference in canalization of size or shape variation

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 2). 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 3). Thus, hypothesis 1 was not rejected for size variation, but it was rejected for shape variation, at least in UM1.

Hypothesis 2: no difference in morphological integration

The variance of the eigenvalue (VE) was significantly greater for EDJ than for OES in UM1, but not in um2 (Figure 4). The greater VEs for EDJ were seen in both UM1 and um2, indicating that EDJ was more integrated than OES. Hypothesis 2 was also rejected only for UM1.

Hypothesis 3: no difference in 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 5). This meant that there was no difference in the extent to which EDJ and OES would be influenced by the selection vector. Hypothesis 3 was not rejected for either both tooth class.

Hypothesis 4: UM1 and um2 share common patterns of canalization, morphological integration, and evolutionary flexibility between the EDJ and the OES

The overall patterns of whether EDJ or OES showed a larger value were common for UM1 and um2 in all of the measurements. However, the results of statistical tests were different for canalization in shape variation and for morphological integration, in which only UM1 showed a significant difference between EDJ and OES. At least in these two cases, hypothesis 4 was rejected.

Discussion

The lack of significant difference in size variation between EDJ and OES in both tooth classes examined here suggests that the strength of canalization on size is almost constant throughout the processes of morphogenesis and the subsequent enamel formation period. A recent developmental study revealed that crown sizes were regulated by intrinsic factors from mesenchymal tissues (Cai et al., 2007). Several dental metrics studies confirmed that tooth crown size was less variable than intercuspal distance and/or cusp size owing to stronger genetic control (Townsend et al., 2003; Harris and Dihn, 2006). Additionally, the spatial relationship with the surrounding tissues, including the maxillary bone and/or other tooth germs, and the available space for tooth growth (Boughner, 2011) may be involved in the canalization of crown size during odontogenesis. The extent of the deviation from mean size in EDJ and OES were not significantly different, and therefore both EDJ size differences and OES size differences among groups being compared can be used as a reliable measure of phylogenetic relatedness.

In the case of UM1, shape variation of OES was greater than that of EDJ. This result suggests that canalization of crown shape may be weakened during the process of enamel formation. Kraus and Jordan (1965) argued that early stages of tooth development are mediated by genes that are more evolutionarily stable than those associated with calcification. Hlusko's (2004) simulation model indicated that enamel thickness could change rapidly under appropriate selective pressure. The present result is in accord with these studies and implies that shape variation is more susceptible to modifications resulting from enamel formation than size variation, which might be likely to cause homoplasy that would confuse phylogenetic reconstructions.

The result of VE analysis showed that EDJ was more integrated than OES in UM1, although the same was not supported statistically in um2. Molar crown morphogenesis is a morphodynamic process in which inductive events and morphogenetic processes act at the same time, and is regulated by interactions between the epithelial and underlying mesenchymal tissues. Cusp initiation and patterning in tooth germ is an iterative process that repeatedly utilizes the same set of genes and signaling pathways, which would lead to

higher morphological integration in EDJ. On the other hand, the pattern of enamel formation is the end product of a sequence proceeding from ameloblast differentiation from the IEE cells, to secretion of protein matrix of amelogenins and enamelines, and finally organization of the enamel crystallites into enamel rods or prisms (Boyde, 1964, 1989). Topological developmental parameters, such as the rate and the duration of enamel apposition and/or ameloblast extension and termination (Simmer et al., 2010), might impact the OES formation, which could lead to weaker morphological integration in OES.

It is predicted that stronger integration between traits acts as a limitation on producing phenotypic variation (Wagner and Altenberg, 1996). The results of the canalization and morphological integration analyses presented here are consistent with this prediction, i.e., the more strongly integrated EDJ shows smaller variability. The regulatory genes expressed during morphogenesis of the tooth are also used in different organs, including hair, pancreas, mammary gland, salivary gland, thymus, vibrissae, and others (Fincham et al., 2000; Jernvall and Jung, 2000). Size and shape of EDJ are thus more likely to be stabilized in order to reduce the risks of negative pleiotropic side effects. The high level of integration in EDJ can be regarded as a relatively rigorous developmental constraint during odontogenesis. Meanwhile, the set of genes that contribute to enamel formation, such as amelogenin gene, enamelin gene, ameloblastin gene, and enamelysin gene, is highly specialized, and can easily modify the OES morphology during the enamel formation process. Morphological change of the OES, which has less developmental constraint, can easily be brought about by neutral evolution by non-natural selective genetic factors such as random genetic drift.

The observed pattern of morphological integration and the results of evolutionary flexibility analyses presented here are not consistent with those of previous studies, in which low levels of integration accompanied high levels of evolvability (Marroig et al., 2009; Porto et al., 2009; Lewton, 2012). The developmental constraints due to canalization and morphological integration act more strongly on the shape of EDJ than on that of OES in UM1, while there is no significant difference in the evolutionary flexibility between EDJ and OES. This may result from the relatively integrated covariance structure of each cusp (for both EDJ and OES). Since the secondary enamel knot that functions as a signaling center and regulates

cuspid formation at the future cuspid tip acts as a “developmental module” (Jernvall and Jung, 2000), it can directly affect the covariance structure of EDJ, and indirectly affect that of the overlying OES. In the case of the human tooth, if the crown covariance structure is divided into individual cuspid units, this pattern cascade mode of cuspid development facilitates the ability to respond to selective challenges (Jernvall and Jung, 2000), and enables the maintenance of a certain level of evolvability at EDJ despite existence of developmental constraints. The comparable level of evolutionary flexibility between EDJ and OES suggests that both of them can be utilized as an equally effective proxy for inferring phylogenetic relationships that would result from selective pressure.

Overall, the difference of each parameter (canalization, morphological integration and evolutionary flexibility) between the EDJ and OES in the present study was greater in UM1 than in um2. The process of enamel formation is more likely to influence crown morphological variability and evolvability in UM1 than in um2, which can be explained by the duration and/or thickness of enamel formation. Compared to UM1, the enamel deposition period of um2 is shorter and the enamel is thinner (Ten Cate, 2012). Therefore enamel formation may exert less influence on shape variation 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.

References

- Alberch P (1982) Developmental constraints in evolutionary processes. In: *Development in evolution* (ed. Bonner JT), pp. 313–332. Berlin and New York: Springer-Verlag.
- Avishai G, Müller R, Gabet Y, Bab I, Zilberman U, Smith P (2004) New approach to quantifying developmental variation in the dentition using serial microtomographic imaging. *Microsc Res Tech* 65, 263–299.
- Bookstein FL (1997) Landmark methods for forms without landmarks: morphometrics of group differences in outline shape. *Med Image Anal* 1, 225–243.
- Boughner JC (2011) Making space for permanent molars in growing baboon (*Papio anubis*) and great ape (*Pan paniscus* and *P. troglodytes*) mandibles: Possible ontogenetic strategies and solutions. *Anat Res Int* doi.org/10.1155/2011/484607.
- Boyde A (1964) The structure and development of mammalian enamel. PhD dissertation. University of London.
- Boyde A (1989) Enamel. In: *Handbook of Microscopic Anatomy, Volume 6: Teeth* (eds. Oksche A and Vollrath L), pp. 309–473. Berlin and New York: Springer-Verlag.
- Butler PM (1956) The ontogeny of molar pattern. *Biol Rev* 31, 30–70.
- Butler PM (1971) Growth of human tooth germs. In: *Dental Morphology and Evolution* (ed. Dahlberg AA), pp. 3–14. Chicago: University of Chicago Press.
- Cai J, Cho SW, Kim JY, Lee MJ, Cha YG, Jung HS (2007) Patterning the size and number of tooth and its cusps. *Dev Biol* 304, 499–507.
- Chernoff B, Magwene PM (1999) Morphological Integration: Forty Years Later. In: *Morphological Integration* (eds. Olsen EC, Miller RL), pp. 319–353. Chicago: University of Chicago Press.
- Cheverud JM (1996) Developmental integration and the evolution of pleiotropy. *Am Zool* 36, 44–50.
- Collard M, Wood B (2000) How reliable are human phylogenetic hypotheses? *Proc Natl Acad Sci U S A* 97, 5003–5006.

- Dahlberg AA (1945) The changing dentition of man. *J Am Dent Assoc* 32, 676–690.
- Dean MC (2000) Progress in understanding hominoid dental development. *J Anat* 197, 77–101.
- Finarelli JA, Clyde WC (2004) Reassessing hominoid phylogeny: evaluating congruence in the morphological and temporal data. *Paleobiol* 30, 614–651.
- Fincham AG, Luo W, Moradian-Oldak J, Paine ML, Snead ML, Zeichner-David M (2000) Enamel biomineralization: the assembly and disassembly of the protein extracellular organic matrix. In: *Development, Function and Evolution of Teeth* (eds. Teaford MF, Meredith-Smith M, Ferguson MWJ), pp. 37–61. Cambridge: Cambridge University Press.
- Grabowski MW (2013) Hominin obstetrics and the evolution of constraints. *Evol Biol* 40, 57–75.
- Gunz P, Mitteroecker P, Bookstein FL (2005) Semilandmarks in three dimensions. In: *Modern Morphometrics in Physical Anthropology* (ed. Slice DE), pp. 73–98. New York: Kluwer Academic/Plenum Publishers.
- Hallgrímsson B, Willmore K, Hall BK (2002) Canalization, developmental stability, and morphological integration in primate limbs. *Am J Phys Anthropol* 35, 131–158.
- Hallgrímsson B, Jamniczky H, Young NM, et al. (2009) Deciphering the palimpsest: Studying the relationship between morphological integration and phenotypic covariation. *Evol Biol* 36, 355–376.
- Hansen TF (2003) Is modularity necessary for evolvability? Remarks on the relationship between pleiotropy and evolvability. *Biosystems* 69, 83–94.
- Harris EF, Dinh DP (2006) Intercusp relationships of the permanent maxillary first and second molars in American whites. *Am J Phys Anthropol* 130, 514–28.
- Hlusko LJ (2004) Integrating the genotype and phenotype in hominid paleontology. *Proc Natl Acad Sci U S A* 101, 2653–2657.
- Hlusko LJ, Suwa G, Kono R, Mahaney MC (2004) Genetics and the evolution of primate enamel thickness: A baboon model. *Am J Phys Anthropol* 124, 223–233.
- Hunter JP, Jernvall J (1995) The hypocone as a key innovation in mammalian evolution. *Proc Natl Acad Sci U S A* 92, 10718–10722.

- Jernvall J, Jung HS (2000) Genotype, phenotype, and developmental biology of molar tooth characters. *Year Phys Anthropol* 43, 171–190.
- Klingenberg CP (2011) MorphoJ: an integrated software package for geometric morphometrics. *Molec Ecol Res* 11, 353–357.
- Korenhof CAW (1960) Morphogenetical Aspects of the Human Upper Molar. Utrecht: Uitgeversmaatschappij Neerlandia.
- Kraus BS (1952) Morphologic relationships between enamel and dentin surfaces of lower first molar teeth. *J Dent Res* 31, 248–256.
- Kraus BS, Jordan RE (1965) The Human Dentition Before Birth. Philadelphia: Lea and Febiger.
- Lande R (1979) Quantitative genetic analysis of multivariate evolution, applied to brain: Body size allometry. *Evolution* 33, 402–416.
- Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. *Evolution* 37, 1210–1226.
- Lewton KL (2012) Evolvability of the primate pelvic girdle. *Evol Biol* 39, 126–139.
- Manly BFJ (1997) Randomization, bootstrap and Monte Carlo methods in biology. London: Chapman & Hall.
- Matsumura H, Domett KM, O'reilly DJW (2011) On the origin of pre-Angkorian peoples: perspectives from cranial and dental affinity of the human remains from Iron Age Phum Snay, Cambodia. *Anthropol Sci* 119, 67–79.
- Maynard Smith J, Burian R, Kauffman S, et al. (1985) Developmental constraints and evolution. *Q Rev Biol* 60, 265–287.
- Marroig G, Shirai L, Porto A, de Oliveira F, De Conto V (2009) The evolution of modularity in the mammalian skull II: Evolutionary consequences. *Evol Biol* 36, 136–148.
- Miller GS (1918) The Piltdown jaw. *Am J Phys Anthropol* 1, 25–52.
- Olejniczak AJ, Gilbert CG, Martin LB, Smith TM, Ulhaas L, Grine FE (2007) Morphology of the enamel-dentine junction in sections of anthropoid primate maxillary molars. *J Hum Evol* 53, 292–301.
- Ortiz A, Skinner MM, Bailey SE, Hublin JJ (2012) Carabelli's trait revisited: an examination

of mesiolingual features at the enamel-dentine junction and enamel surface of Pan and Homo sapiens upper molars. *J Hum Evol* 63, 586–596.

Pilbrow V (2006) Population systematics of chimpanzees using molar morphometrics. *J Hum Evol* 51, 646–662.

Porto A, de Oliveira FB, Shirai LT, de Conto V, Marroig G (2009) The evolution of modularity in the mammalian skull I: Morphological integration patterns and magnitudes. *Evol Biol* 36, 118–135.

R Development Core Team (2011) R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. <http://cran.R-project.org>.

Rohlf FJ, Slice D (1990) Extensions of the Procrustes method for the optimal superimposition of landmarks. *Syst Zool* 39, 40–59.

Sakai K, Kanazawa E (1999) Morphological traits on the dentino-enamel junction of lower deciduous molar series. In: *Dental morphology 1998: Proceedings of the 11th international symposium on dental morphology* (eds. Mayhall J, Heikkinen T), pp. 167–178. Oulu: Oulu University Press.

Saunders SR, Mayhall JT (1982) Developmental patterns of human morphological traits. *Archs Oral Biol* 27, 45–49.

Simmer JP, Papagerakis P, Smith CE, et al. (2010) Regulation of dental enamel shape and hardness. *J Dent Res* 89, 1024–1038.

Simons EL, Pilbeam D (1972) Hominoid paleoprimatology. In: *The Functional and Evolutionary Biology of Primates* (ed. Tuttle R), pp. 36–62. Chicago: Aldine-Atherton.

Skinner MM, Wood BA, Hublin JJ (2009) Protostylid expression at the enameledentine junction and enamel surface of mandibular molars of Paranthropus robustus and Australopithecus africanus. *J Hum Evol* 56, 76–85.

Skinner MM, Evans A, Smith T, et al. (2010) Brief Communication: Contributions of enamel-dentine junction shape and enamel deposition to primate molar crown complexity. *Am J Phys Anthropol* 142, 157–163.

Skinner MM, Gunz P (2010) The presence of accessory cusps in chimpanzee lower molars is consistent with a patterning cascade model of development. *J Anat* 217, 245–253.

- Smith P, Gomorri JM, Spitz S, Becker J (1997) Model for the examination of evolutionary trends in tooth development. *Am J Phys Anthropol* 102, 283–294.
- Smith P, Gomori JM, Shaked R, Haydenblit R, Joskowicz L (2000) A computerized approach to reconstruction of growth patterns in hominid molar teeth. In: *Proceedings of the 11th International Symposium on Dental Morphology* (eds. Mayhall J, Heikkinen T), pp. 388–397. Oulu: Oulu University Press.
- Smith P, Avishai G, Muller R, Gabet Y (2011) Computerized reconstruction of prenatal growth trajectories in the dentition: Implications for the taxonomic status of Neandertals. In: *Continuity and discontinuity in the peopling of Europe: One hundred fifty years of Neanderthal study* (eds. Condemi S, Weniger G-C), pp. 165–173. New York: Springer Science+Business Media B.V.
- Sokal RR, Braumann CA (1980) Significance tests for coefficients of variation and variability profiles. *Syst Zool* 29, 50–66.
- Suzuki M, Sakai T (1973) Occlusal surface pattern of the lower molars and the second deciduous molar among living Polynesians. *Am J Phys Anthropol* 39, 305–315.
- Ten Cate AR (2012) *Oral Histology: Development, Structure and Function*, 8th Edition. St. Louis: C.V. Mosby Company.
- Townsend G, Richards L, Hughes T (2003) Molar intercusp dimensions: genetic input to phenotypic variation. *J Dent Res* 82, 350–355.
- Villmoare B, Fish J, Jungers W (2011) Selection, morphological integration, and strepsirrhine locomotor adaptations. *Evol Biol* 38, 88–99.
- Wagner GP (1984) On the eigenvalue distribution of genetic and phenotypic dispersion matrices: Evidence for a nonrandom organization of quantitative character variation. *J Math Biol* 21, 77–95.
- Wagner GP, Altenberg L (1996) Complex adaptations and the evolution of evolvability. *Evolution* 50, 967–976.
- Wagner GP, Booth G, Bagheri-Chaichian H (1997) A population genetic theory of canalization. *Evolution* 51, 329–347.
- Willmore KE, Young N, Richtsmeier JT (2007) Phenotypic variability: its components,

measurement and underlying developmental processes. *Evol Biol* 34, 99–120.

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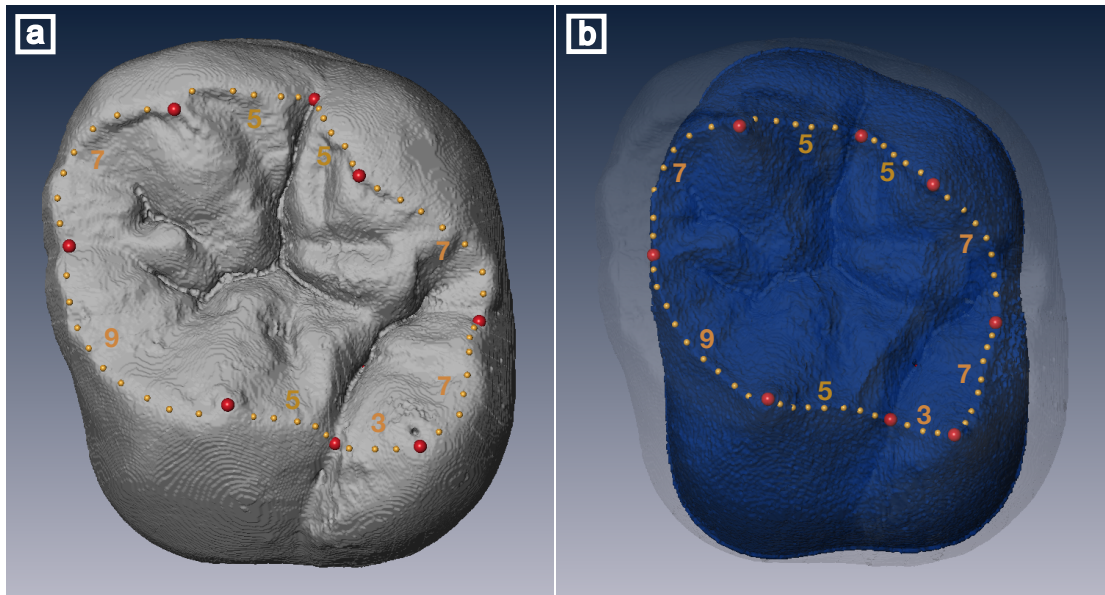


Figure 1. Digital image of permanent maxillary first molar crown (lingual view). (a) OES ridge curve digitized on the OES. (b) EDJ ridge curve digitized on the EDJ surface. Red circles are landmarks, and yellow circles are semi-landmarks. Numbers appended to each section of the ridge curve refer to the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

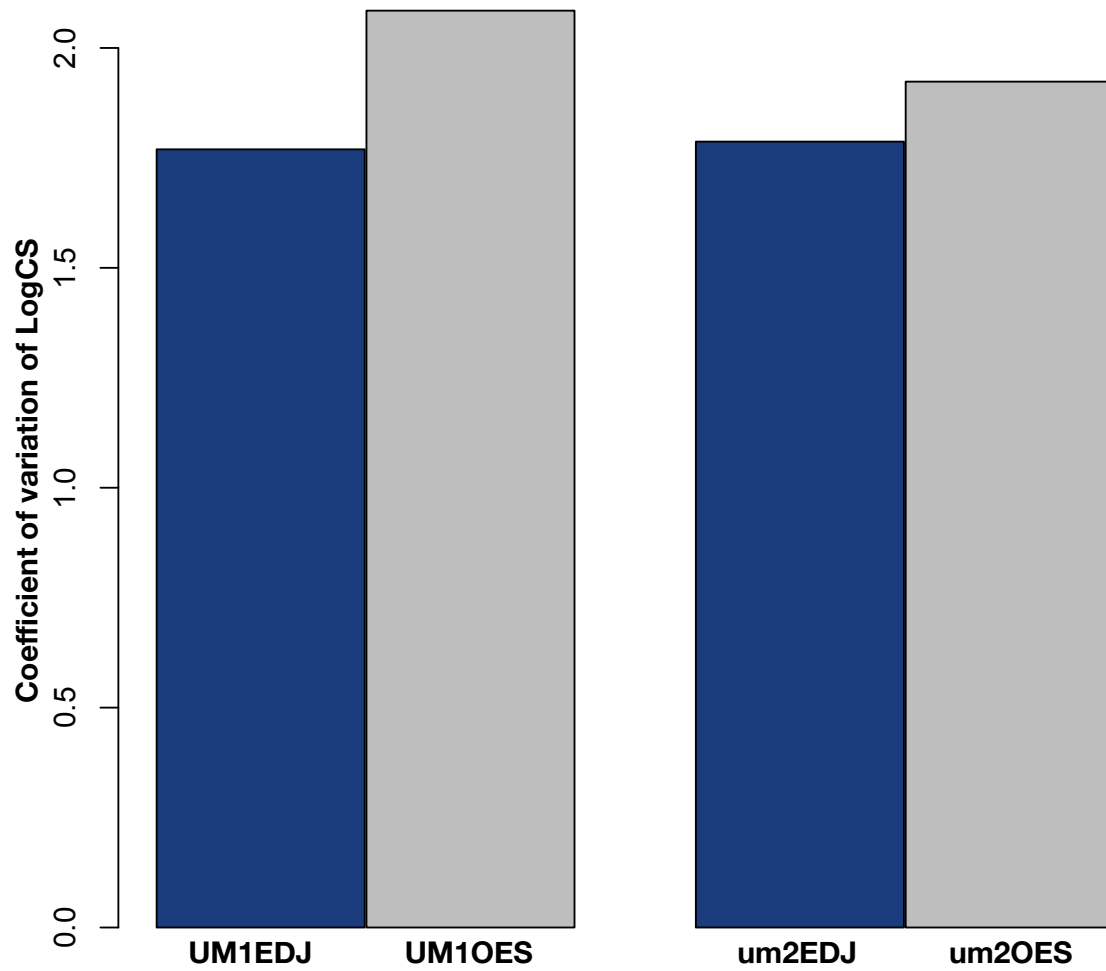


Figure 2. Bar graph showing the size variation for four configurations (UM1EDJ, UM1OES, um2EDJ and um2OES). Significance test for coefficient of variation for LogCS among them reveals that there is no significant difference ($P>0.05$).

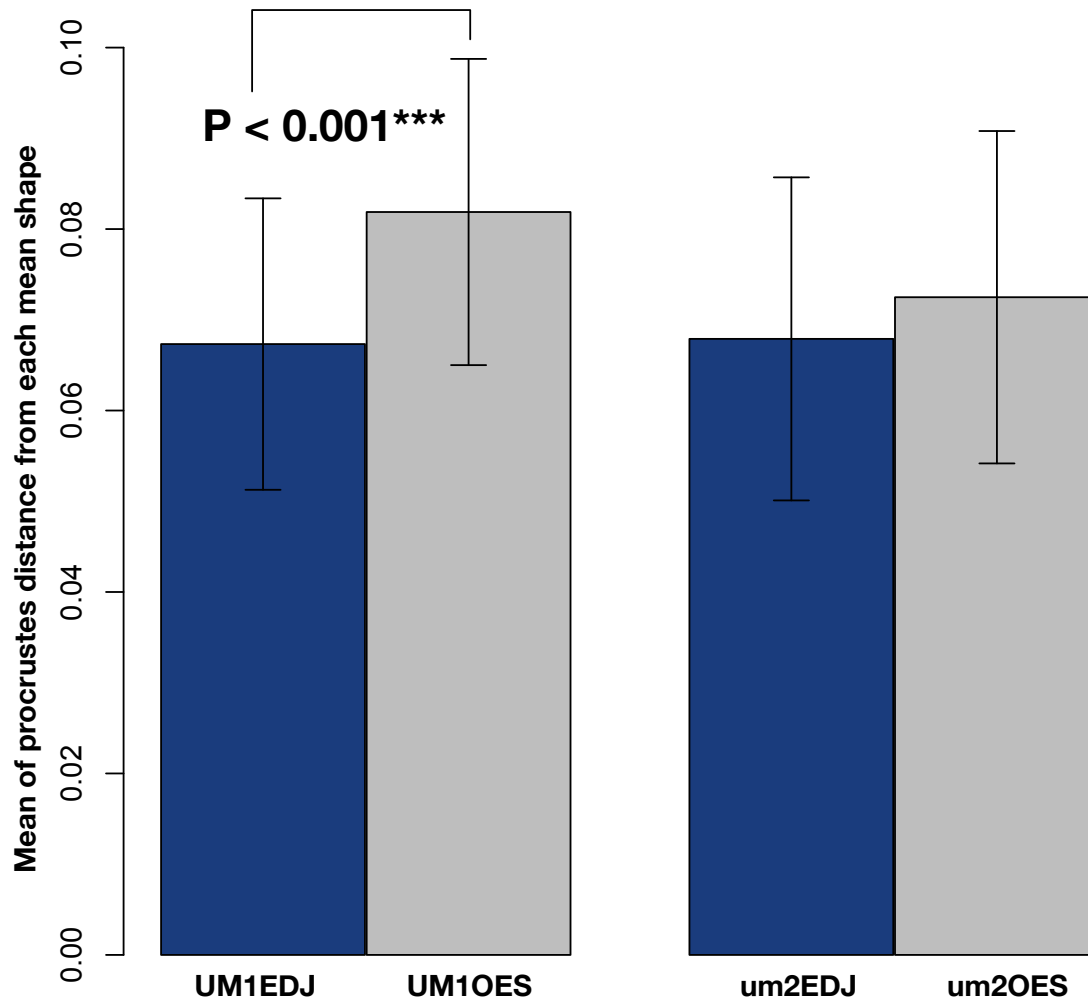


Figure 3. Bar graph showing mean of procrustes distance from each mean shape for shape variance of four configurations (UM1EDJ, UM1OES, um2EDJ and um2OES), and the error bars show standard deviations. The Kruskal-Wallis test reveals a significant difference among them ($P < 0.001$). A nonparametric multiple-comparison test between EDJ and OES within the same tooth class reveals that the difference is highly significant in UM1 ($P < 0.001$).

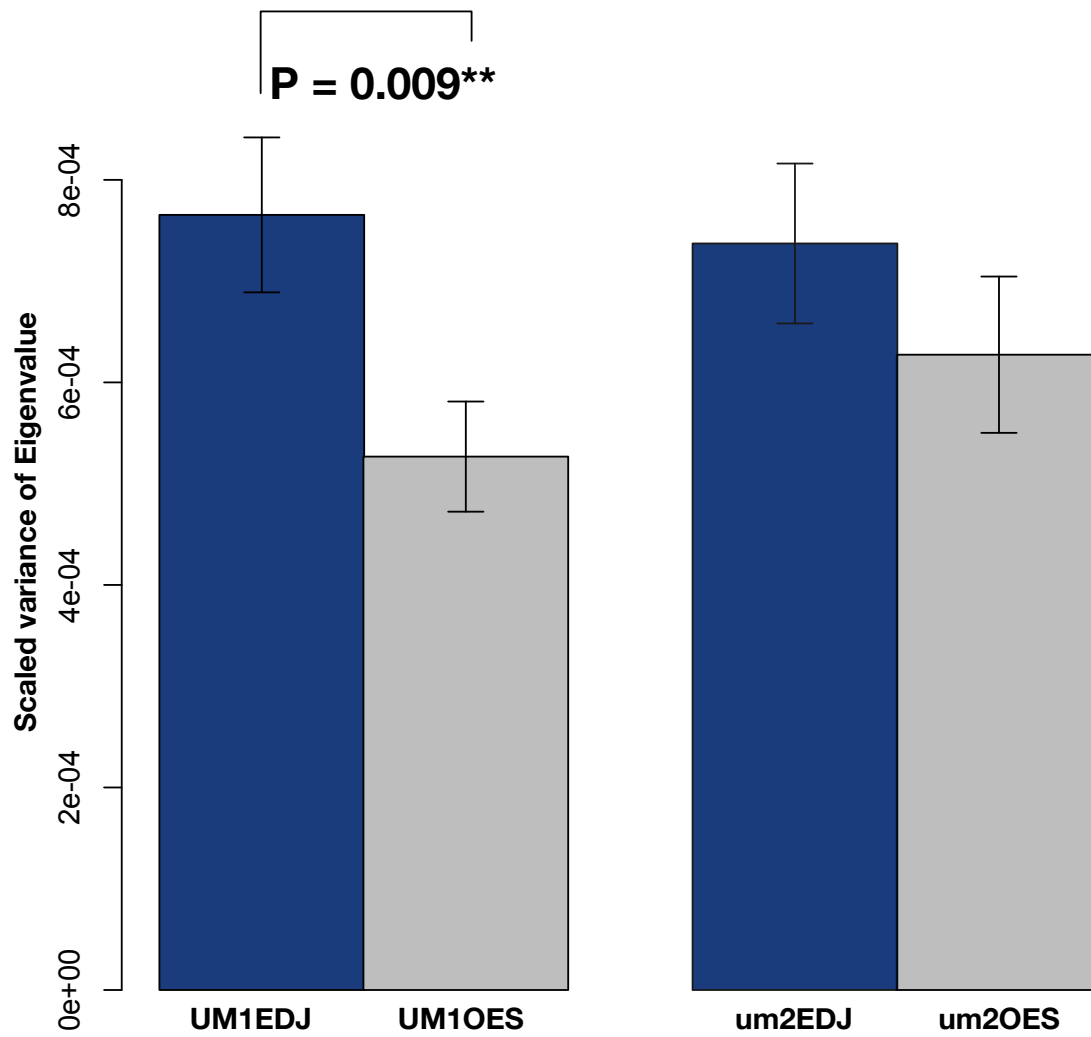


Figure 4. Bar graph showing the scaled variances of eigenvalue for morphological integration for four configurations (UM1EDJ, UM1OES, um2EDJ and um2OES). The error bars shown are standard deviations obtained by resampling the original datasets with replacement 1000 times. Bootstrap tests between EDJ and OES within the same tooth class reveal that the difference is highly significant only in UM1 ($P=0.009$).

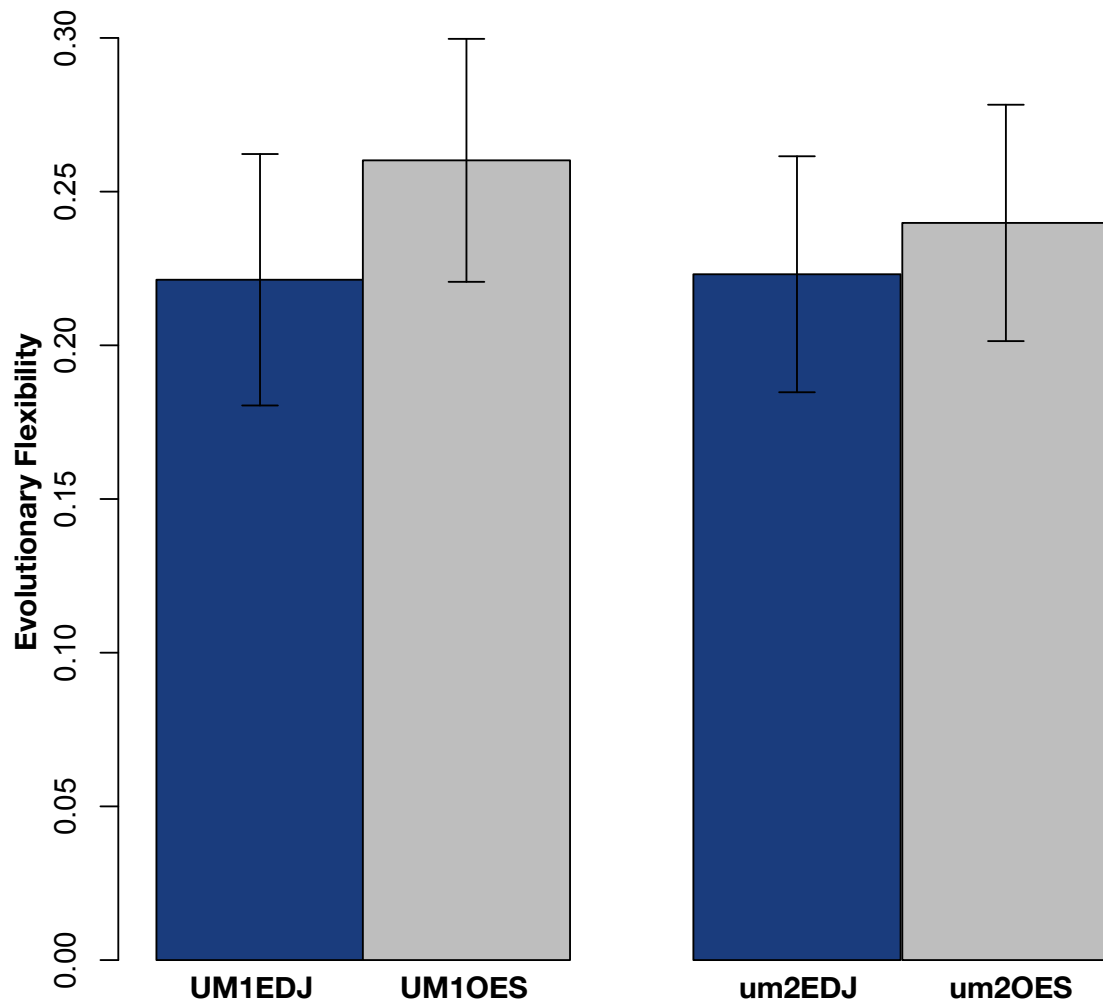


Figure 5. Bar graph showing the evolutionary flexibility for four configurations (UM1EDJ, UM1OES, um2EDJ and um2OES). The error bars shown are standard deviations obtained by resampling the original datasets with replacement 1000 times. Bootstrap tests between EDJ and OES within the same tooth class reveal that there is no significant difference ($P>0.05$).

Chapter 4: Discussion

This thesis focused on the morphological variability of the enamel-dentin junction (EDJ) and outer-enamel surface (OES) in human maxillary first permanent molar and second deciduous molar. Whole crown morphology including internal and external structures was analyzed by high resolution computed tomography. Reconstructed surfaces were analyzed quantitatively by geometric morphometric methods. These analyses explored patterns of morphological variation in relation to the order of cusp initiation through the odontogenesis, and compared properties of variability and evolvability between EDJ and OES. Then, insights of the developmental factors responsible for morphological variability were discussed. Two specific questions are addressed in order to elucidate morphological variability in human maxillary molars. These specific questions and the major conclusions drawn from the analyses can be summarized as follows.

How the patterns of morphological variability in human molar teeth are structured during odontogenesis?

Patterns of size and shape variability among cusps were examined at total and each particular crown component. The results showed that the pattern of size variability was caused by temporal factors, such as the order of cusp initiation and the duration from the beginning of mineralization to the completion of crown formation, whereas shape variability might be affected by both topographic and temporal factors.

What kinds of the properties of morphological variability structured by odontogenetic process are existed in human molar teeth, and how they affect evolutionary change?

The strength of two components of phenotypic variability: canalization and morphological integration, plus the relevant evolutionary flexibility were compared between EDJ and OES. The EDJ morphology was more canalized and integrated than OES. It indicated that canalization and morphological integration acted as developmental constraints at EDJ. On the other hand, EDJ and OES had the comparable level of evolvability, possibly because the pattern cascade mode of cusp development would cause a cusp-divided covariance structure.

Previous studies noted that EDJ preserves primitive features such as vestigial cusplets, ridges, and cingulua that are not apparent at OES (Korenhoff, 1961; Kraus and

Jordan, 1965; Sakai and Hanamura, 1973; Corruccini, 1987). A high level of developmental constraint acted at EDJ might have suppressed evolutionary changes. Similarly, the morphology of um2 has been considered to be more conservative from an evolutionary perspective than that of UM1 (Dahlberg, 1945; Butler, 1956, 1971; Suzuki and Sakai, 1973; Saunders and Mayhall, 1982), which is consistent with findings of this dissertation that in um2 there are small difference in properties of variability between EDJ and OES.

This study revealed that size variation of human UM1 and um2 was smaller than that of shape and stabilized through the odontogenetic process. Therefore, it was conformed with PCM. The presence or absence of cusps can be explained under the framework of PCM, and tooth crown size that can be responsible for the number of cusps and expression of crown features is considered to have a great influence on the tooth crown form (Skinner et al., 2008, 2009; Hunter et al., 2010; Skinner and Gunz, 2010; Ortiz et al., 2012). UM1 and um2 are morphologically more stable than the other teeth belonged to the same molarization field (Butler, 1939; Margetts and Brown, 1978; Farmer and Townsend, 1993; Scott and Turner, 1997; Liversidge and Molleson, 1999). This suggested that these teeth used in this study might be likely to have inherent small size variation. Therefore, phylogenetic studies need to care for properties of variability of the sample.

Natural selection can act on EDJ for fundamental cusp pattern and morphology involved in resistance to occlusal loads, and on OES for outer surface form for proper mastication. Albeit higher degree of developmental constraint at EDJ, both EDJ and OES had a comparable level of evolvability, possibly due to shared cusp-divided covariance structure. Indeed, the iterative activation of secondary enamel knots may be inherent mechanism of evolvability, as the enamel knots can be understood as developmental modules (Jernvall and Jung, 2000). Modularity of development has been suggested to be one of the mechanisms of evolvability because it facilitates local and independent changes in morphology (Wagner and Altenberg, 1996; Gerhart and Kirschner, 1997; Lewton, 2012). Developmentally, multicusped teeth in mammals are formed by the repeated appearance of secondary enamel knots (Jernvall, 1995; Jernvall and Thesleff, 2000, 2012). Polly (1998) proposed “a general model for the evolution of tooth development”, and the common model for tooth morphogenesis is able to

form a tooth-like 3D morphology closely resembling those of mouse, vole and other species by simulation analysis (Salazar-Ciudad, 2008; Salazar-Ciudad and Jernvall, 2002, 2010). Are patterns of variability and evolvability of human maxillary molars observed in the present study common at least in those of hominoids that share the same occlusal morphology?

The distribution pattern of enamel on the tooth crown is different among extant hominoids, which may reflect differences in dietary patterns and developmental mechanisms (Schwartz, 2000). Evolvability used in the present analysis, i.e., evolutionary flexibility, demonstrates the capacity to respond to selection in any direction in phenotypic space (Marroig et al., 2009). These simulated directions in phenotypic space may not reflect selective pressures that actual hominoid populations have experienced (Rolian, 2009). Thus, comparative analysis among hominoid species is required for a refined understanding of the relationships between morphological variability, natural selection, and phylogeny.

Through a comparison between UM1 with um2, this dissertation provided insights how the differences in developmental process affect on patterns of variation among cusps and morphological variability. These results raise a research question what kinds of developmental parameter affects on morphological variability. A direct approach is organ culture experiments of tooth germ. Tinkering with signaling molecules mediating interactions during tooth development or using mutant mice molars will provide new information on how the developmental process generates morphological variability. It requires a feed back loop from experimental studies for further understanding of the relationships between developmental process and tooth morphology, which will connect genotype and phenotype and address the elucidation of the mechanisms of tooth morphological evolution.

References

- Butler PM (1939) Studies of the mammalian dentition-differentiation of the post-canine dentition. *Proc Zool Soc Lond B* 109, 1–36.
- Butler PM (1956) The ontogeny of molar pattern. *Biol Rev* 31, 30–70.
- Butler PM (1971) Growth of human tooth germs. In: *Dental Morphology and Evolution* (ed. Dahlberg AA), pp. 3–14. Chicago: University of Chicago Press.

- Corruccini RS (1987) The dentinoenamel junction in primates. *Int J Primatol* 8, 99–114.
- Dahlberg AA (1945) The changing dentition of man. *J Am Dent Assoc* 32, 676–690.
- Farmer V, Townsend GC (1993) Crown size variability in the deciduous dentition of South Australian children. *Am J Hum Biol* 5, 681–690.
- Gerhart J, Kirschner M (1997) Cell, embryos and evolution. Oxford: Blackwell Scientific.
- Hunter JP, Guatelli-Steinberg D, Weston TC, Durner R, Bestsinger TK (2010) Model of tooth morphogenesis predicts Carabelli feature expression, size, and symmetry in humans. *PLoS One* 5, 7 e11844.
- Jernvall J (1995) Mammalian molar cusp patterns: Developmental mechanisms of diversity. *Acta Zool Fenn* 198, 1–61.
- Jernvall J, Jung HS (2000) Genotype, phenotype, and developmental biology of molar tooth characters. *Year Phys Anthropol* 43, 171–190.
- Jernvall J, Thesleff I (2000) Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech Dev* 92, 19–29.
- Jernvall J, Thesleff I (2012) Tooth shape formation and tooth renewal: evolving with the same signals. *Development* 139, 3487–3497.
- Korenhof CAW (1961) The enamel-dentine border: a new morphological factor in the study of the (human) molar pattern. *Proc Koninkl Nederl Acad Wetensch* 64B, 639–664.
- Kraus BS, Jordan RE (1965) The Human Dentition Before Birth. Philadelphia: Lea and Febiger.
- Lewton KL (2012) Evolvability of the primate pelvic girdle. *Evol Biol* 39, 126–139.
- Liversidge HM, Molleson TI (1999) Deciduous tooth size and morphogenetic fields in children from Christ Church, Spitalfields. *Arch Oral Biol* 44, 7–13.
- Margetts B, Brown T (1978) Crown diameters of the deciduous teeth in Australian aboriginals. *Am J Phys Anthropol* 48, 493–502.
- Marroig G, Shirai L, Porto A, de Oliveira F, De Conto V (2009) The evolution of modularity in the mammalian skull ii: Evolutionary consequences. *Evol Biol* 36, 136–148.
- Ortiz A, Skinner MM, Bailey SE, Hublin JJ (2012) Carabelli's trait revisited: An examination of mesiolingual features at the enamel-dentine junction and enamel surface of Pan and Homo

sapiens upper molars. *J Hum Evol* 63, 586–596.

Polly PD (1998) Variability, selection, and constraints: development and evolution in viverravid (Carnivora, Mammalia) molar morphology. *Paleobiology* 24, 409–429.

Rolian C (2009) Integration and evolvability in primate hands and feet. *Evol Biol* 36, 100–117.

Sakai T, Hanamura H (1973) A morphological study of enamel-dentin border on the Japanese dentition: VII. General conclusion. *J Anthropol Soc Nippon* 81, 87–102.

Salazar-Ciudad I. (2008) Tooth morphogenesis in vivo, in vitro, and in silico. *Curr Top Dev Biol* 81, 341–71.

Salazar-Ciudad I, Jernvall J (2002) A gene network model accounting for development and evolution of mammalian teeth. *Proc Natl Acad Sci U S A* 99, 8116–8120.

Salazar-Ciudad I, Jernvall J (2010) A computational model of teeth and the developmental origins of morphological variation. *Nature* 464, 583–586.

Saunders SR, Mayhall JT (1982) Developmental patterns of human morphological traits. *Archs Oral Biol* 27, 45–49.

Schwartz GT (2000) Taxonomic and functional aspects of the patterning of enamel thickness distribution in extant large-bodied hominoids. *Am J Phys Anthropol* 111, 221–244.

Scott GR, Turner II CG (1997) *The Anthropology of Modern Human Teeth*. Cambridge: Cambridge University Press.

Skinner MM, Gunz P (2010) The presence of accessory cusps in chimpanzee lower molars is consistent with a patterning cascade model of development. *J Anat* 217, 245–253.

Skinner MM, Wood BA, Boesch C, Olejniczak AJ, Rosas A, Smith TM, Hublin JJ (2008) Dental trait expression at the enamel-dentine junction of lower molars in extant and fossil hominoids. *J Hum Evol* 54, 173–186.

Skinner MM, Wood BA, Hublin JJ (2009) Protostylid expression at the enamel-dentine junction and enamel surface of mandibular molars of *Paranthropus robustus* and *Australopithecus africanus*. *J Hum Evol* 56, 76–85.

Suzuki M, Sakai T (1973) Occlusal surface pattern of the lower molars and the second deciduous molar among living Polynesians. *Am J Phys Anthropol* 39, 305–315.

Wagner GP, Altenberg L (1996) Complex adaptations and the evolution of evolvability.
Evolution 50, 967–976.

Appendix: Molar sample used in this dissertation including site information

Specimen ID	Tooth	Period ¹	Site	Side
KUMA326	UM1	Jomon	Yoshigo shell mound, Aichi Pref.	R
KUMA274	UM1	Jomon	Yoshigo shell mound, Aichi Pref.	L
KUMA268	UM1	Jomon	Yoshigo shell mound, Aichi Pref.	L
KUMA52	UM1	Jomon	Tsugumo shell mound, Okayama Pref.	L
KUMA276	UM1	Jomon	Yoshigo shell mound, Aichi Pref.	L
KUMA334	UM1	Jomon	Yoshigo shell mound, Aichi Pref.	L
KUMA402	UM1	Jomon	Yoshigo shell mound, Aichi Pref.	L
KUMA457	UM1	Jomon	Yoshigo shell mound, Aichi Pref.	L
SKT100	UM1	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT223-1	UM1	Edo	Sakai-kanogo-toshi, Osaka Pref.	R
SKT223	UM1	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT499	UM1	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
Ht32	UM1	Edo	Hitotsubashi, Tokyo Metro.	R
Ht120	UM1	Edo	Hitotsubashi, Tokyo Metro.	L
Ht199	UM1	Edo	Hitotsubashi, Tokyo Metro.	R
Ht208	UM1	Edo	Hitotsubashi, Tokyo Metro.	R
Ht272	UM1	Edo	Hitotsubashi, Tokyo Metro.	R
Ht278	UM1	Edo	Hitotsubashi, Tokyo Metro.	R
Ht367	UM1	Edo	Hitotsubashi, Tokyo Metro.	L
YMMB27-7-2	UM1	Medieval	Chusei-shudan-Bochi, Kanagawa Pref.	L
YMMB52-5	UM1	Medieval	Chusei-shudan-Bochi, Kanagawa Pref.	L
YH11	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	R
YMMB293	UM1	Medieval	Chusei-shudan-Bochi, Kanagawa Pref.	L
YMMB333	UM1	Medieval	Chusei-shudan-Bochi, Kanagawa Pref.	L
YH148	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	R
YH200E	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	R
YH211A	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	R
YH232A	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	L
YH244	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	L
YH248	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	L
YH1076	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	R
YH1189B	UM1	Medieval	Yuigahama-minami, Kanagawa Pref.	L
CK45	UM1	Edo	Chokoji, Aichi Pref.	R
CK50	UM1	Edo	Chokoji, Aichi Pref.	L
CK72	UM1	Edo	Chokoji, Aichi Pref.	L
CK10	UM1	Edo	Chokoji, Aichi Pref.	R
CK174	UM1	Edo	Chokoji, Aichi Pref.	L
CK152	UM1	Edo	Chokoji, Aichi Pref.	R
CK169	UM1	Edo	Chokoji, Aichi Pref.	R
CK66	UM1	Edo	Chokoji, Aichi Pref.	R
CK159	UM1	Edo	Chokoji, Aichi Pref.	R

CK146	UM1	Edo	Chokoji, Aichi Pref.	L
CK112	UM1	Edo	Chokoji, Aichi Pref.	L
CK68	UM1	Edo	Chokoji, Aichi Pref.	L
CK140	UM1	Edo	Chokoji, Aichi Pref.	R
CK51	UM1	Edo	Chokoji, Aichi Pref.	R
CK9	UM1	Edo	Chokoji, Aichi Pref.	R
CK122	UM1	Edo	Chokoji, Aichi Pref.	L
CK186	UM1	Edo	Chokoji, Aichi Pref.	R
CK29	UM1	Edo	Chokoji, Aichi Pref.	R
CKUN12-1	UM1	Edo	Chokoji, Aichi Pref.	R
CK105	UM1	Edo	Chokoji, Aichi Pref.	L
CK13	UM1	Edo	Chokoji, Aichi Pref.	R
CK151	UM1	Edo	Chokoji, Aichi Pref.	R
CK183	UM1	Edo	Chokoji, Aichi Pref.	R
CKUN9	UM1	Edo	Chokoji, Aichi Pref.	R
CK166	UM1	Edo	Chokoji, Aichi Pref.	R
KUMA297	um2	Jomon	Yoshigo shell mound, Aichi Pref.	L
KUMA327	um2	Jomon	Yoshigo shell mound, Aichi Pref.	R
KUMA332	um2	Jomon	Yoshigo shell mound, Aichi Pref.	L
KUMA430	um2	Jomon	Yoshigo shell mound, Aichi Pref.	L
KUMA551	um2	Jomon	Yoshigo shell mound, Aichi Pref.	R
SKT158	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	R
SKT202	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT252	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	R
SKT261	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT271	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT312	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT393	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	R
SKT410	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	R
SKT452	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	R
SKT453B	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT491	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT535	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	L
SKT561	um2	Edo	Sakai-kanogo-toshi, Osaka Pref.	R
YMMB313	um2	Medieval	Chusei-shudan-Bochi, Kanagawa Pref.	R
YMMB193	um2	Medieval	Chusei-shudan-Bochi, Kanagawa Pref.	R
YMMB173	um2	Medieval	Chusei-shudan-Bochi, Kanagawa Pref.	L
Ht273	um2	Edo	Hitotsubashi, Tokyo Metro.	R
Ht231	um2	Edo	Hitotsubashi, Tokyo Metro.	L
Ht203	um2	Edo	Hitotsubashi, Tokyo Metro.	R
Ht201	um2	Edo	Hitotsubashi, Tokyo Metro.	R
Ht153	um2	Edo	Hitotsubashi, Tokyo Metro.	L
Ht196	um2	Edo	Hitotsubashi, Tokyo Metro.	R
YH44A	um2	Medieval	Yuigahama-minami, Kanagawa Pref.	R
YH26D	um2	Medieval	Yuigahama-minami, Kanagawa Pref.	R
YH176B	um2	Medieval	Yuigahama-minami, Kanagawa Pref.	R

YH131A	um2	Medieval	Yuigahama-minami, Kanagawa Pref.	L
YH130	um2	Medieval	Yuigahama-minami, Kanagawa Pref.	R
CK37	um2	Edo	Chokoji, Aichi Pref.	R
CK81	um2	Edo	Chokoji, Aichi Pref.	R
CK118	um2	Edo	Chokoji, Aichi Pref.	L
CK184	um2	Edo	Chokoji, Aichi Pref.	L
CK5	um2	Edo	Chokoji, Aichi Pref.	R
CK18	um2	Edo	Chokoji, Aichi Pref.	R
CK133	um2	Edo	Chokoji, Aichi Pref.	L
CK73	um2	Edo	Chokoji, Aichi Pref.	L
CK162	um2	Edo	Chokoji, Aichi Pref.	L
CK59	um2	Edo	Chokoji, Aichi Pref.	R
CK86	um2	Edo	Chokoji, Aichi Pref.	R
CK83	um2	Edo	Chokoji, Aichi Pref.	L
CK119	um2	Edo	Chokoji, Aichi Pref.	R
CK2	um2	Edo	Chokoji, Aichi Pref.	R
CKUN2	um2	Edo	Chokoji, Aichi Pref.	R
CK133	um2	Edo	Chokoji, Aichi Pref.	R

1. Jomon (14500-300 BC); Medieval (13-15C AD); Edo (17-19C AD).

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