Published in final edited form as:

Clin Genet. 2011 September ; 80(3): 265–272. doi:10.1111/j.1399-0004.2010.01529.x.

A novel g.-1258G>A mutation in a conserved putative regulatory element of *PAX9* is associated with autosomal dominant molar hypodontia

GA Mendoza-Fandino[a], **JM Gee**[a,c], **S Ben-Dor**[d], **C Gonzalez-Quevedo**[a], **K Lee**[e], **Y Kobayashi**[f], **J Hartiala**[a,b], **RM Myers**[f,*], **SM Leal**[e], **H Allayee**[a,b], and **PI Patel**[a,c]

[a]Institute for Genetic Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

[b]Department of Preventive Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA

[c]Center for Craniofacial Molecular Biology, Herman Ostrow School of Dentistry of USC, University of Southern California, Los Angeles, CA, USA

[d]Bioinformatics and Biological Computing Unit, Weizmann Institute of Science, Tel Aviv, Israel

[e]Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX, USA

[f]Department of Genetics, Stanford University School of Medicine, Stanford, CA, USA

Abstract

Mutations in the transcription factor PAX9 which plays a critical role in the switching of odontogenic potential from the epithelium to the mesenchyme during tooth development cause autosomal dominant non-syndromic hypodontia primarily affecting molars. Linkage analysis on a family segregating autosomal dominant molar hypodontia with markers flanking and within *PAX9* yielded a maximum multipoint LOD score of 3.6. No sequence variants were detected in the coding or 5′- and 3′-untranslated regions (UTRs) of *PAX9*. However, we identified a novel g.-1258G>A sequence variant in all affected individuals of the family but not in the unaffected family members or in 3088 control chromosomes. This mutation is within a putative 5′-regulatory sequence upstream of *PAX9* highly conserved in primates, somewhat conserved in ungulates and carnivores but not conserved in rodents. Bioinformatics analysis of the sequence determined that there was no abolition or creation of a putative binding site for known transcription factors. Based on our previous findings that haploinsufficiency for PAX9 leads to hypodontia, we postulate that the g.-1258G>A variant reduces the expression of PAX9 which underlies the hypodontia phenotype in this family.

Corresponding author: Pragna I. Patel, PhD, Institute for Genetic Medicine, University of Southern California, 2250 Alcazar Street, IGM-240, Los Angeles, CA 90033, USA. Tel.: +1 (323)442 2751; Fax: +1 (323) 442 2764; pragna@usc.edu.
*Current address: HudsonAlpha Institute for Biotechnology, Huntsville, AL, USA

Conflict of interest
Nothing to declare.

Keywords

hypodontia; molars; multiple-species conserved sequence; mutation; *PAX9*; tooth agenesis

Hypodontia (OMIM 106600) is the common generalized term used to describe congenitally missing teeth and is one of the most frequent developmental anomalies in humans (1). Teeth most commonly found missing are the third molars (20%), second premolars (3.4%), and maxillary lateral incisors (2.2%), with the absence of six or more teeth much less frequent (0.08% to 1.1 %) (2). Genetic linkage studies on non-syndromic hypodontia have so far identified three genes underlying this condition: *MSX1* (3), *PAX9* (4) and *AXIN2* (5)

The transcription factors Pax9 and Msx1, which are known to be essential for the switch in odontogenic potential from the epithelium to the mesenchyme, are expressed in the dental mesenchyme at the initiation stage of tooth development (6, 7). Pax9 interacts with Msx1 at both the gene and protein levels, which enhances the ability of Pax9 to transactivate *Msx1* and *Bmp4* expression during tooth development (8). Furthermore, Pax9 is needed for the expression of *Msx1* in the mesenchyme during tooth morphogenesis (8). Studies in mouse models have shown that Pax9 is required at multiple stages during odontogenesis and changes of *Pax9* expression levels have a direct consequence on dental patterning (9, 10).

To date, 11 unique mutations at the PAX9 locus have been described in families segregating hypodontia of primarily molar teeth. They include frame-shift (4, 11, 12), insertion (12, 13), missense (12) and nonsense mutations (14, 15) as well as deletion of the entire gene (16–18). Here, we describe a novel mutation in a putative regulatory element of the gene which was not present in over 3000 normal chromosomes, supporting the hypothesis that this mutation causes hypodontia in this family.

Materials and methods

Subjects

Written informed consent was obtained from all members of family DEN29 who participated in this study. This study was approved by the Institutional Review Board of the University of Southern California (IRB protocol HS-045053). A detailed family history was used to construct the pedigree of family DEN29 and confirmed through personal interviews with family members. Affection status was assigned through personal examination and/or review of dental records and radiographs obtained from the subjects' dental practitioner. Blood or buccal cell samples were obtained from 17 family members of whom 11 were affected, 5 were unaffected, and 1 was of unknown status.

Linkage analysis

Genomic DNA was extracted from whole blood using the PureGene kit (Gentra System, Minneapolis, MN). Genomic DNA from buccal cells was extracted using kits from either Epicentre (Madison, WI) or Oragene (DNA Genotek, Toronto, Canada) following the manufacturers' recommended protocols. Linkage analysis of the *PAX9* locus was performed using a single nucleotide polymorphism (SNP) within exon 2 and three microsatellite

markers D14S1462, D14S1463, and D14S1464 located 39, 49 and 72 kb upstream of the *PAX9* gene, respectively (16). The SNP in exon 2 was genotyped by sequencing the polymerase chain reaction (PCR) product obtained with primers hPAX9ex2aF and hPAX9ex2aR (4) as described below. Microsatellite markers were PCR-amplified with a FAM-labeled 5′ primer, and genotyped using an ABI3100 genetic analyzer and GENOTYPER version 3.7 (Applied Biosystems, Foster City, CA). Two-point linkage analysis was carried out using the MLINK program of the FASTLINK computer package (19). Conservative estimates of gene frequency (1/10,000), phenocopy rate (1/1000) and penetrance (95%) were used in the calculation of LOD scores.

DNA sequencing

Previously designed primers flanking the coding regions of exons 1–4 of the *PAX9* gene were used to sequence all exons (4) in two affected family members, IV:6 and V:3. Primers to amplify and sequence the 5.5 kb region upstream of the translational start site of the *PAX9* gene were designed using PRIMER3 version 2.0 (primer sequences available upon request). PCR-amplified products were subjected to dye-terminator sequencing using BigDye Terminator 3.1 fluorescent sequencing technology on an ABI3100 genetic analyzer (Applied Biosystems). DNA sequencing results were analyzed using SEQUENCING ANALYSIS Software version 3.7 (Applied Biosystems).

Genotyping of g.-1258G>A sequence variant

Initial genotyping of the *PAX9* g.-1258G>A sequence variant within family DEN29 was performed using a restriction assay for the *Rsa*I site that is destroyed by this sequence variant. Primers pax9NCS1cF (AGACTTAGTCCCAAGACAGTGGAC) and pax9NCS1cR (AGATTCCGATTCTAAAGAGTGGTG) were used to amplify the 580 bp region surrounding the variant. The amplified product was digested with 1U of *Rsa* I for 1 h at 37° C and analyzed on a 2% agarose gel. The A-allele yields fragments of 328 and 252 bp, while the G allele yields fragments of 328, 140 and 112 bp. To analyze the *PAX9* g.-1258G>A sequence variant within a control race-matched population of unrelated individuals, a TaqMan genotyping assay was designed (Applied Biosystems). The sequences of primers and probes were as follows: Forward primer – GTTCTGCCTACTTCTGAATTTTCACTTTTA; reverse primer – TTCTCCACAACCCTAACTGCAAAT; probe – AAAAGCATACG[A/G]TACACTT. The assay was performed using the manufacturer's recommended protocol on an ABI Prism 7900HT sequence detection system (Applied Biosystems) with ABgene ABsolute™ QPCR ROX (500nM) mix (Rochester, NY).

Bioinformatics analysis of sequence

The putative promoter region (chr14:36,198,723– 36,20l,050, UCSC assembly hg18), including the g.-1258G>A variant, was analyzed using MATINSPECTOR (http://www.genomatix.de) (20) with default parameters to identify potential transcription factor binding sites that are abolished or acquired in the presence of the variant A-allele.

Results

Clinical findings

We ascertained a four-generation European-American family designated DEN29, in which non-syndromic hypodontia segregated in an autosomal dominant manner (Fig. 1a). Affected individuals were missing molars in all four quadrants, as well as some premolars and lateral incisors (Fig. 1b). On average, 12 permanent teeth were missing in affected individuals. There were no apparent abnormalities of nails, skin, hair, or sweat glands.

Linkage analysis

In order to determine if the locus underlying non-syndromic hypodontia in family DEN29 was linked to the *PAX9* gene locus, we sampled 11 affected individuals, five unaffected, and one individual of unknown status and conducted linkage analysis. Linkage analysis was performed with three microsatellite markers (D14S1462, D14S1463, and D14S1464) upstream of *PAX9* (16) and a SNP within exon 2. There were no recombination events between these markers and the disease locus. Consequently, all 11 affected individuals shared the same haplotype. A maximum two-point LOD score of 3.6 under a dominant model was obtained with all markers, showing significant evidence for linkage of hypodontia to the *PAX9* genomic region. These results strongly implicated a mutation in *PAX9* as a possible cause of the hypodontia in family DEN29

Identification of PAX9 g.-1258G>A sequence variant

In order to identify a possible mutation within the *PAX9* gene, the coding sequence and the 5′- and 3′-UTR were amplified using primers flanking the exons and sequenced. We compared the sequence of three affected family members to the reference sequence of *PAX9* in the National Center for Biotechnology Information (NCBI) Gen-Bank database (Build 36.1). However, all identified sequence variants in these regions were either present in dbSNP or present in unaffected family members (data not shown), suggesting that they were not the underlying genetic cause of hypodontia in this family. We next compared the 5′-flanking sequence of the human *PAX9* gene orthologous sequence in the rhesus, marmoset, dog, horse, mouse and rat genomes using VISTA tools version 2.0 (21) (Fig. 3a). A 5.5 kb region immediately upstream of the initiation codon contained several multiple-species conserved sequences (MCS) and thus, the entire region was selected for sequence analysis in two affected individuals, IV:6 and V:3. A G to A sequence variant was identified 1258 bp upstream of the translational start codon of the *PAX9* gene *(g.-1258G>A)* (Fig. 2a). Affected and unaffected family members of DEN29 were screened for the presence of the g.-1258G>A sequence variant using a restriction assay targeting an *Rsa*I site that is destroyed in the presence of the variant A-allele. This variant was present only in the affected family members (Fig. 2b).

To further ascertain whether the g.-1258G>A variant was truly associated with the disease in DEN29 family and not present in other control individuals of the same ethnicity as DEN29, 1544 unrelated Caucasian individuals were screened using a TaqMan genotyping assay. None of the 3088 control chromosomes carried the g.-1258G>A variant (95% confidence interval 0–0.0012), strongly suggesting that this was the causal mutation in family DEN29.

Functional characterization of the g.-1258G>A mutation

We previously reported that haploinsufficiency for PAX9 due to the deletion of PAX9 gene causes hypodontia of molar teeth (16), similar to that seen in members of family DEN29. Given this finding, we reasoned that the mutation in family DEN29 could lead to reduced *PAX9* expression and that this might be caused by abolition or creation of a transcription factor binding site or disruption of a key nucleotide within a regulatory element such as an enhancer. We were unable to reliably compare *PAX9* mRNA levels between affected and unaffected individuals in lymphocytes or lymphoblasts, which were the only sources of RNA available from participants, as the gene is expressed at extremely low levels in these cells. We also conducted bioinformatics analysis of the region encompassing the g.-1258G>A variant using MATINSPECTOR (20) to determine if the mutation abolished or created a transcription factor binding site. This analysis did not reveal disruption of any transcription factor binding sites. The presence of an A instead of a G did suggest the presence of a binding site for DMRT3 (Doublesex and mab-3 related transcription factor 3) (22); however, close examination of the sequence and comparison to the consensus sequence reported by Murphy et al. and validated by them through *in vitro* binding studies (22) revealed that the region bearing the variant was missing a strongly selected T at position −8 of the consensus and hence, not a good match for DMRT3.

Discussion

In the present study, linkage analysis of a family segregating non-syndromic molar hypodontia in an autosomal dominant manner identified a mutation at the *PAX9* locus as the underlying cause of the hypodontia. Direct sequencing of the coding regions of *PAX9* failed to identify any putative causative sequence variants in affected individuals. However, sequencing of the region encompassing 5.5 kb upstream of the *PAX9* translational start site identified a C>T variant 1258 bp upstream (g.-1258G>A) that is located in an MCS (Fig. 3b). The variant A-allele was present only in affected family members and not in any of the unaffected family members or in 1544 unrelated control individuals of the same ethnicity.

Two *Pax9* transcripts have been reported in the mouse with a major transcript whose start site corresponds to that initially reported and a minor transcript that includes 333 bp of additional sequence at the 5′ end comprising an exon they have termed exon 0 (23). There are four ESTs that support the existence of exon 0 in humans as well (accession numbers: DB233208, DB196800, DB218464, DA667732). The g.-1258G>A variant we have identified is located between exons 0 and 1 and is upstream of the promoter that presumably drives transcription of the major transcript. Comparison of the sequence of the 5′-flanking region of *PAX9* in various mammalian species using VISTA tools (21) determined that the region immediately upstream of the initiation codon bears stretches of sequence that is conserved across several vertebrates (Fig. 3a). A comparison of a 60 bp segment bearing the g.-1258G>A variant in family DEN29 shown in Fig. 3b reveals that this sequence is highly conserved only in primate, somewhat lesser conserved in ungulate, canine and feline genomes and least conserved in the mouse and rat genomes. While this observation is intriguing, its significance with respect to the differences between primate dentition and that in other species cannot be discerned based on our current knowledge of tooth development.

The presence of the g.-1258G>A variant in an MCS element in the putative *PAX9* promoter region suggested that it may function as a transcriptional regulatory element. We were unable to directly determine if the mutation resulted in lower levels of *PAX9* transcript due to the fact that the only source of cells from subjects bearing the mutation were lymphocytes and lymphoblasts and the very low levels of *PAX9* mRNA in these cells made any comparisons unreliable. Although bioinformatics analysis using MATINSPECTOR did not identify any consensus binding sequence for a transcription factor at the site of the mutation, it did suggest that the mutation created a consensus sequence for the binding site of DMRT3 which is a member of the proteins related to *Drosophila* doublesex and *Caenorhabditis elegans* mab-3 transcription factors. However, close examination of the consensus binding sequence identified by Murphy et al. (22) and verified by *in vitro* binding experiments indicated that this is not likely to be a true binding site. *In situ* hybridization studies have shown that DMRT3 has a restricted embryonic expression profile that is conserved in chicken and mouse embryos (24) and that DMRT3 is expressed primarily in the forebrain, neural tube and nasal placode of both species. We attempted to determine whether the variant resides within a putative enhancer element by cloning a 1265 bp fragment (−184 to −1449) bearing either the G (wild-type) or A (mutant) at −1258 into the vector pGL4.11 (Promega Corp., Madison, WI), and transfecting it into several cell lines [MCF-7 (25); MDPC-23 (26); HepG2 (27)] but we were unable to detect reporter gene expression from these vectors although reporter expression was observed with a positive control. Further experimentation *in vivo* is required to determine the functional consequences of the g.-1258G>A mutation on gene expression.

The vast majority of disease-causing mutations in mendelian diseases are within the coding sequence and manifest as missense, frame-shift, or nonsense mutations. This observation is also true for *PAX9* mutations leading to hypodontia. Our study of family DEN29 now suggests that a mutation in a conserved upstream regulatory region of *PAX9*, which presumably leads to reduced mRNA and consequently protein levels, can also lead to hypodontia. This notion is supported by segregation analysis as well as a survey of 3088 control chromosomes, which taken together, provide strong support for the causative role of the g.-1258G>A mutation in family DEN29. However, given that *PAX9* is a developmentally expressed gene, we were limited in our ability to test the functional consequences of the variant in the context of the developing tooth or other organs where *PAX9* is normally expressed during development. While it is theoretically possible that the g.-1258G>A variant is in linkage disequilibrium with the actual disease-causing variant which we did not identify through our sequencing efforts, the absence of this variant in >3000 chromosomes supports its causative role in hypodontia.

Acknowledgements

We are grateful to the members of family DEN29 for their participation in this study. We thank Trevor Pemberton, Larry Kedes and Joe Hacia for valuable discussions and Sunju Choi for assistance with figures. This work was supported by NIH grant DE014102 (P.I.P.). This investigation was partly conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 (RR10600–01, CA62528–01, RR14514– 01) from the NCRR, NIH. No researchers involved in this study report a conflict of interest.

References

1. De Coster PJ, Marks LA, Martens LC et al. Dental agenesis: genetic and clinical perspectives. J Oral Pathol Med 2009: 38: 1–17. [PubMed: 18771513]
2. Schalk-van der Weide Y, Steen WHA, Bosman F. Distribution of missing teeth and tooth morphology in patients with oligodontia. J Dent Child 1992: 59: 133–140.
3. Vastardis H, Karimbux N, Guthua SW et al. A human MSX1 homeodomain missense mutation causes selective tooth agenesis. Nat Genet 1996: 13: 417–421. [PubMed: 8696335]
4. Stockton DW, Das P, Goldenberg M et al. Mutation of PAX9 is associated with oligodontia. Nat Genet 2000: 24: 18–19. [PubMed: 10615120]
5. Lammi L, Arte S, Somer M et al. Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. Am J Hum Genet 2004: 74: 1043–1050. [PubMed: 15042511]
6. Jowett AK, Vainio S, Ferguson MWJ et al. Epithelial-mesenchymal interactions are required for msx 1 and msx 2gene expression in the developing murine molar tooth. Development 1993: 117: 461–470. [PubMed: 8101167]
7. Peters H, Neubuser A, Kratochwil K et al. Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. Genes Dev 1998: 12: 2735–2747. [PubMed: 9732271]
8. Ogawa T, Kapadia H, Feng JQ et al. Functional consequences of interactions between Pax9 and Msx1 genes in normal and abnormal tooth development. J Biol Chem 2006: 281: 18363. [PubMed: 16651263]
9. Kist R, Watson M, Wang X et al. Reduction of Pax9 gene dosage in an allelic series of mouse mutants causes hypodontia and oligodontia. Hum Mol Genet 2005: 14: 3605–17. [PubMed: 16236760]
10. Kist R, Greally E, Peters H. Derivation of a mouse model for conditional inactivation of Pax9. Genesis 2007: 45: 460–464. [PubMed: 17610273]
11. Frazier-Bowers SA, Guo DC, Cavender A et al. A novel mutation in human PAX9 causes molar oligodontia. J Dent Res 2002: 81: 129–133. [PubMed: 11827258]
12. Das P, Hai M, Elcock C et al. Novel missense mutations and a 288-bp exonic insertion in PAX9 in families with autosomal dominant hypodontia. Am J Med Genet 2003: 118: 35–42.
13. Mostowska A, Biedziak B, Trzeciak WH. A novel mutation in PAX9 causes familial form of molar oligodontia. Eur J Hum Genet 2006: 14: 173–179. [PubMed: 16333316]
14. Nieminen P, Arte S, Tanner D et al. Identification of a nonsense mutation in the PAX9 gene in molar oligodontia. Eur J Hum Genet 2001: 9: 743–746. [PubMed: 11781684]
15. Hansen L, Kreiborg S, Jarlov H et al. A novel nonsense mutation in PAX9 is associated with marked variability in number of missing teeth. Eur J Oral Sci 2007: 115: 330–333. [PubMed: 17697174]
16. Das P, Stockton DW, Bauer C et al. Haploinsufficiency of PAX9 is associated with autosomal dominant hypodontia. Hum Genet 2002: 110: 371–376. [PubMed: 11941488]
17. Devos D, Vuillaume I, de Becdelievre A et al. New syndromic form of benign hereditary chorea is associated with a deletion of TITF-1 and PAX-9 contiguous genes. Mov Disord 2006: 21: 2237–2240. [PubMed: 17044090]
18. Guala A, Falco V, Breedveld G et al. Deletion of PAX9 and oligodontia: a third family and review of the literature. Int J Paediatr Dent 2008: 18: 441–445. [PubMed: 18445003]
19. Cottingham RWJ, Idury RM, Schaffer AA. Faster sequential genetic linkage computations. Am J Hum Genet 1993: 53: 252–263. [PubMed: 8317490]
20. Cartharius K, Frech K, Grote K et al. MatInspector and beyond: promoter analysis based on transcription factor binding sites. Bioinformatics 2005: 21: 2933–2942. [PubMed: 15860560]
21. Mayor C, Brudno M, Schwartz JR et al. VISTA: visualizing global DNA sequence alignments of arbitrary length. Bioinformatics 2000: 16: 1046–1047. [PubMed: 11159318]
22. Murphy MW, Zarkower D, Bardwell VJ. Vertebrate DM domain proteins bind similar DNA sequences and can heterodimerize on DNA. BMC Mol Biol 2007: 8: 58. [PubMed: 17605809]

23. Santagati F, Gerber JK, Blusch JH et al. Comparative analysis of the genomic organization of Pax9 and its conserved physical association with Nkx2-9 in the human, mouse, and pufferfish genomes. Mamm Genome 2001: 12: 232–237. [PubMed: 11252173]

24. Smith CA, Hurley TM, McClive PJ et al. Restricted expression of DMRT3 in chicken and mouse embryos. Gene Expr Patterns 2002: 2: 69–72. [PubMed: 12617839]

25. Soule HD, Vazguez J, Long A et al. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 1973: 51: 1409–1416. [PubMed: 4357757]

26. Sun ZL, Fang DN, Wu XY et al. Expression of dentin sialo-protein (DSP) and other molecular determinants by a new cell line from dental papillae, MDPC-23. Connect Tissue Res 1998: 37: 251–261. [PubMed: 9862225]

27. Aden DP, Fogel A, Plotkin S et al. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 1979: 282: 615–616. [PubMed: 233137]

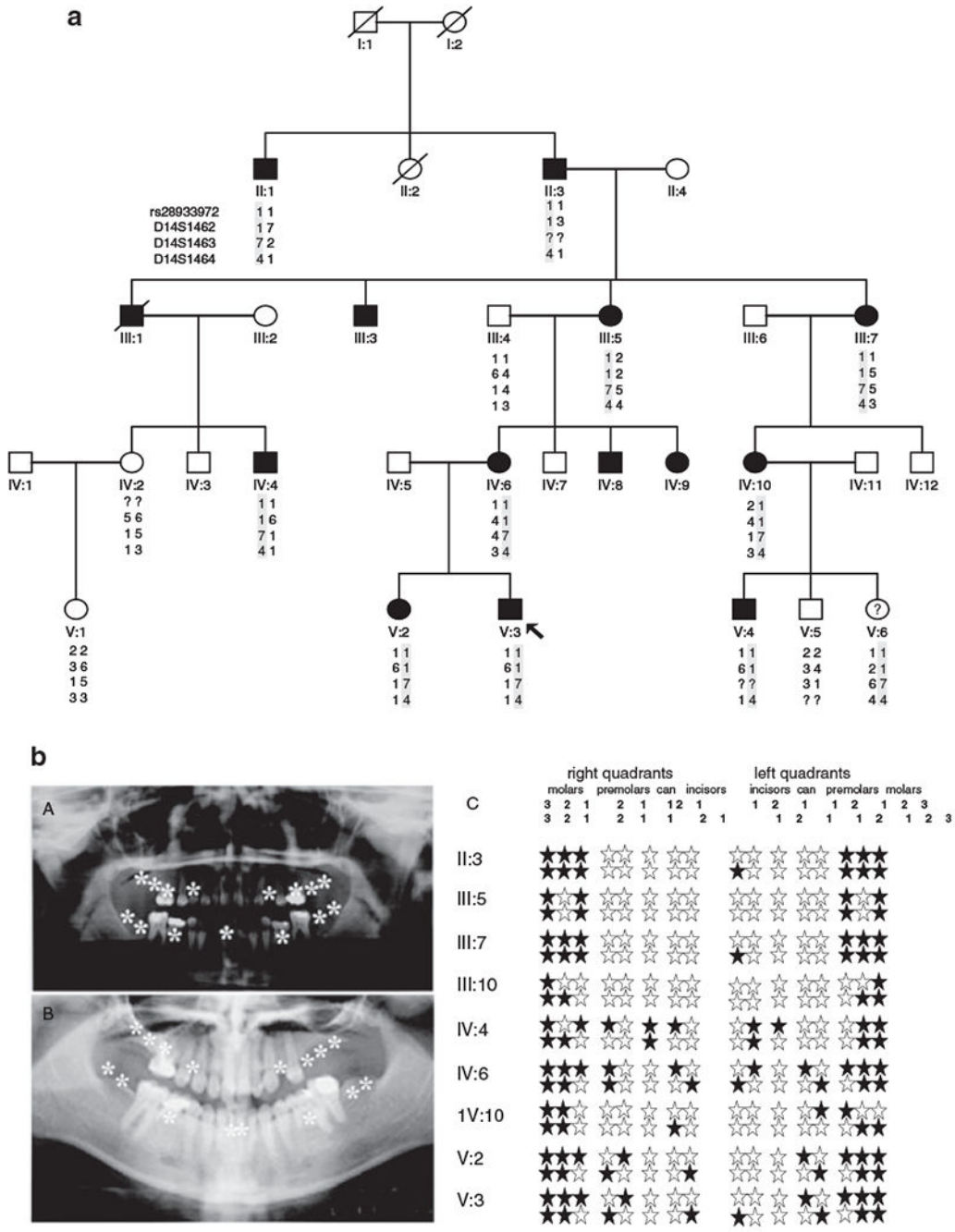

Fig. 1.

(**a**) Pedigree of family DEN29 with haplotypes for a SNP within (rs28933972) and microsatellite markers (D14SA1462, D14S1463, D14S1464) near the PAX9 locus. The shaded haplotype is that segregating with the hypodontia phenotype. (**b**) Panoramic radiographs of individuals V:2 (A) and V:3 (B) showing absence of most maxillary and mandibular permanent teeth; (C) synopsis of the permanent dentition in each of four quadrants in representative affected individuals. Blackened-in stars represent absent teeth.

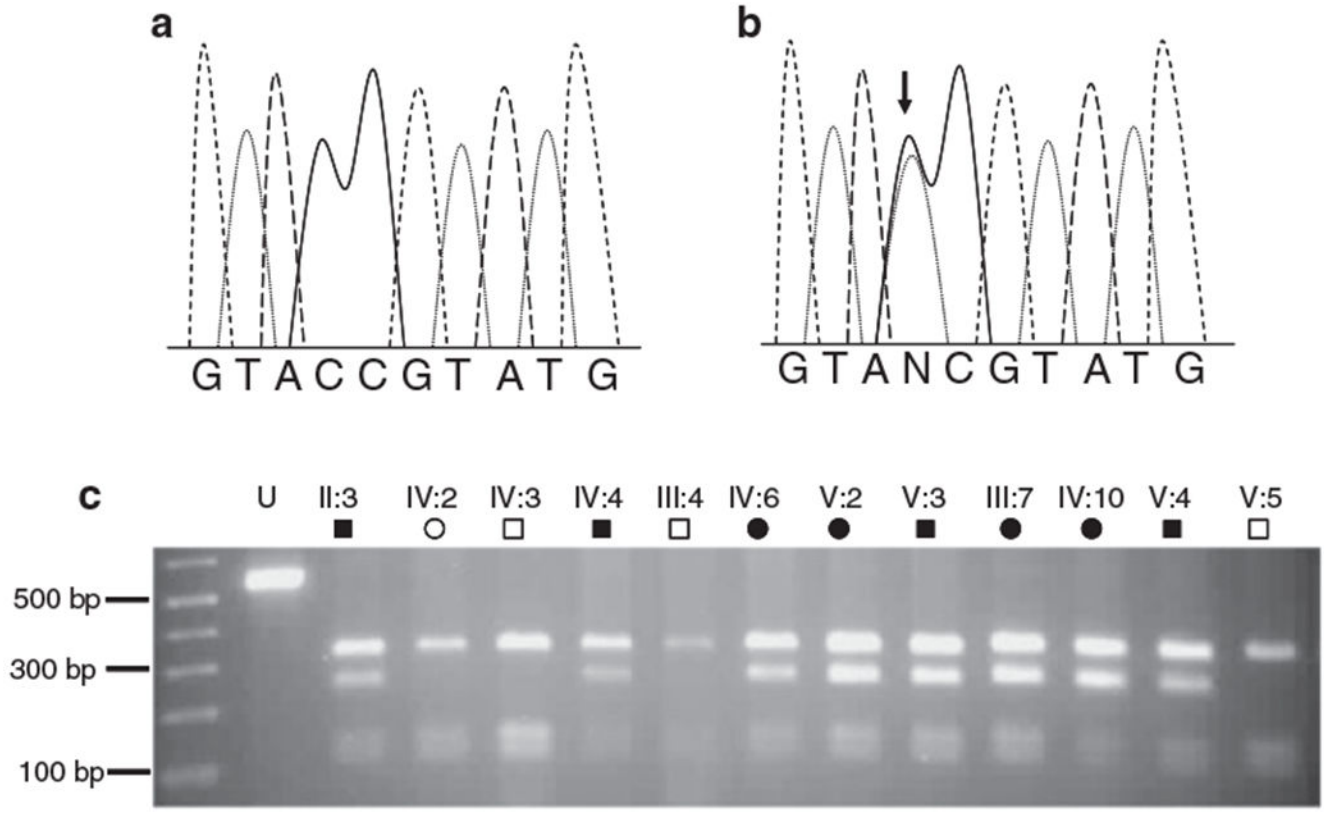

Fig. 2.

DNA sequence chromatograms from a control (**a**) and an affected individual (**b**) from pedigree DEN29 showing the g.-1258G>A sequence variant on the non-coding strand. (**c**) Segregation of the g.-1258G>A variant in DEN29. The g.-1258G>A variant in DEN29 destroyed a cleavage site for *Rsa*I. Amplification of a 580-bp PCR product bearing the variant was followed by digestion with *Rsa* I. The A-allele yielded fragments of 328 and 252 bp, while the G allele yielded fragments of 328, 140 and 112 bp.

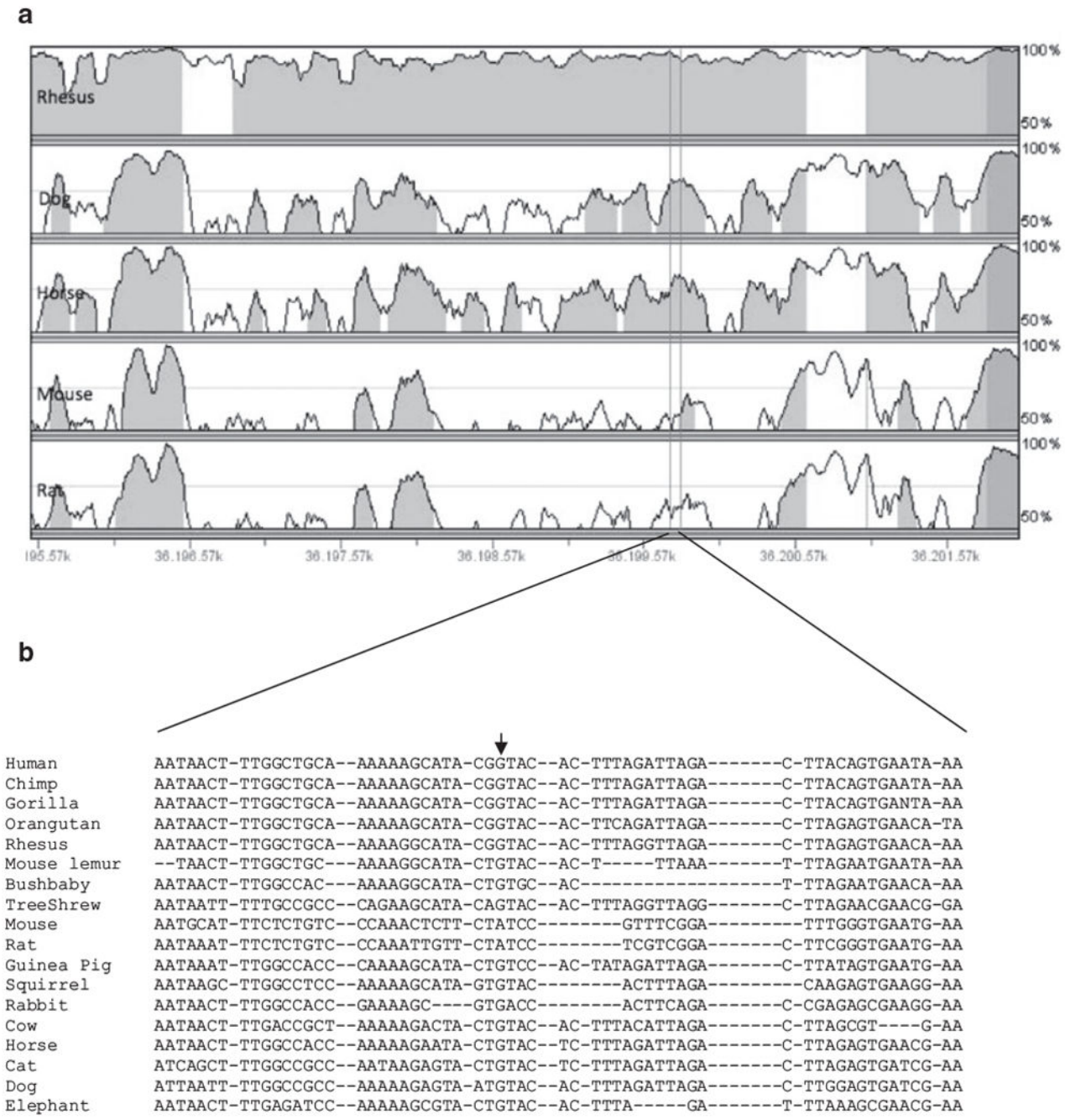

Fig. 3.

(**a**) Sequence alignments of the 6.5 kb genomic interval (chr14:36,195,524 – 36,202,049 on the NCBI build 34 human assembly) containing exons 0, 1 and 2 of the PAX9 gene with orthologous counterparts from representative members of rhesus, dog, horse, mouse, and rat lineages. These are shown as SLAGAN-derived vista representations. The level of conservation (vertical axis) is displayed against the coordinates of the human sequence (horizontal axis). Conserved regions above the level of 70%/100 bp are highlighted under the curve, with dark gray indicating a conserved non-coding region, charcoal gray, a

conserved exon, and light gray, an untranslated region. The vertical lines within the vista plot represent a 60 bp segment bearing the g.-1258G>A variant. (**b**) Multiple-species comparison of a 60 bp segment bearing the g.-1258G>A variant identified by the arrow above the alignment. The multiple alignments were obtained from the UCSC browser where they were generated using two methods, phastCons and phyloP from the PHAST package.