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1   **A de novo *EDA*-variant in a litter of shorthaired standard Dachshunds with X-linked**

2   **hypohidrotic ectodermal dysplasia**

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11   Running title: **A de novo *EDA*-variant in Dachshunds**

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Abstract

In this study, we present a detailed phenotype description and genetic elucidation of the first case of X-linked hypohidrotic ectodermal dysplasia in the shorthaired standard Dachshund. This condition is characterized by partial alopecia, missing and malformed teeth and a lack of eccrine sweat glands. Clinical signs including dental X-raying and histopathological findings were consistent with an ectodermal dysplasia. Pedigree analysis supported an X-recessive mode of inheritance. Whole-genome sequencing of one affected puppy and his dam identified a 1-basepair deletion within the *ectodysplasin-A* gene (CM000039.3:g.54509504delT, PRJEB27789). Sanger sequencing of further family members confirmed the PRJEB27789-variant. Validation in all available family members, 37 unrelated shorthaired standard Dachshunds, 128 Dachshunds from all other breeds and samples from 34 dog breeds revealed the PRJEB27789 variant to be private for this family. Two heterozygous females showed very mild alopecia but normal dentition. Since the dam is demonstrably the only heterozygous animal in the ancestry of the affected animals, we assume that the PRJEB27789-variant arose in the germline of the granddam or in an early embryonic stage of the dam. In conclusion, we detected a very recent de-novo *EDA* mutation causing X-linked hypohidrotic ectodermal dysplasia in the shorthaired standard Dachshund.

Introduction

X-linked hypohidrotic ectodermal dysplasia (XLHED) is a hereditary defect that causes malformations of structures that emerge from the ectoderm, including hair, teeth and sweat glands (Söderholm and Kaitila 1985; Clarke 1987; Kere et al. 1996; Itin 2014). XLHED manifests in humans with alopecia, a lack of sweat glands and possibly other exocrine glands, oligodontia and conically shaped teeth (Clarke et al. 1987; Cluzeau et al. 2011). The affected

43 individuals therefore suffer from hypohidrosis, which can lead to hyperthermia (Mills 1968).

44 They are also affected with decreased mucociliary clearance, predisposing to respiratory tract

45 infections, decreased lacrimal function and dry eye, and problems with mastication (Beahrs et

46 al. 1971; Söderholm and Kaitila 1985; Clarke et al. 1987; Gilgenkrantz et al. 1989; Johnson et

47 al. 2002; Casal et al. 2007). In humans, XLHED is the most frequent form of ectodermal

48 dysplasias (Kere et al. 1996). Ectodermal dysplasias (ED) are a group of genetically

49 heterogeneous diseases. XLHED is caused by alterations in the *ectodysplasin-A (EDA)* gene

50 (Kere et al. 1996), but there are also autosomal dominant, recessive and unknown mutations

51 that cause an ED phenotype (Pinheiro and Freire-Maia 1994; Munoz et al. 1997; Ho et al.

52 1998; Monreal et al. 1999). In humans, only four genes (*EDA, ectodysplasin-A receptor*

53 *(EDAR), EDAR-associated death domain (EDARADD)*, and *wingless-type MMTV integration*

54 *site family, member 10A (WNT10A)*) are causative for 90% of hypo-/anhidrotic ED cases

55 (Cluzeau et al. 2011).

56 *EDA* is located on the X-chromosome. It has several transcripts and protein isoforms. In

57 humans, the two isoforms EDA-A1 and EDA-A2 interact with EDAR and ectodysplasin-A2

58 receptor (EDA2R, formerly XEDAR), respectively (Yan et al. 2000). EDAR can activate

59 nuclear factor kappa-B (NF-κB), c-Jun N-terminal kinase (JNK) and cell death pathways

60 (Kumar et al. 2001; Koppinen et al. 2001). The EDAR-activation of the NF-κB pathway is

61 needed for the development of skin appendages (Mikkola and Thesleff 2003; Schmidt-Ullrich

62 et al. 2006; Cui and Schlessinger 2006).

63 Many ED variants were identified in different species. More than 80 causal mutations are

64 described in humans (Huang et al. 2015). Cases in other species include mice (Srivastava et

65 al. 1997), cattle (Drögemüller et al. 2001), horse (Ramzan et al. 2001), fish (Kondo et al.

66 2001) and dogs. ED phenotypes were shown for the dog breeds Bichon Frisé (Grieshaber et

67 al. 1986), Labrador Retriever (Kunkle 1984), Miniature Poodle (Selmanowitz et al. 1970b),

68   Whippet (Thomsett 1961), Cocker Spaniel (Kral and Schwartzman 1964), Basset Hound

69   (Chastain and Swayne 1985), mixed breeds of miniature Pinscher and Pekingese (Moura and

70   Cirio 2004), German Shepherd (Casal et al. 1997) and mixed breed dogs (Waluk et al. 2016).

71   ED phenotypes have been suspected in the breeds Belgian Shepherd, Beagle, Rottweiler,

72   Yorkshire Terrier, French Bulldog and Lhasa Apso (Miller et al. 2013). In those latter dog

73   breeds, the authors state that it was not always possible to determine whether the dogs

74   exclusively had alopecia or expressed further ectodermal defects, too. The hairless dog breeds

75   Xoloitzcuintle and Chinese crested dog also exhibit an ED phenotype, as they frequently have

76   missing teeth. The hairlessness in these breeds is caused by an autosomal dominant defect in

77   the *FOXI3* gene (Drögemüller et al. 2008). The clinical signs of XLHED-affected dogs are

78   almost identical to those of affected humans (Casal et al. 2007). Treatment of the disease for

79   mice is possible using prenatal intravenous administration of recombinant EDA (Gaide and

80   Schneider 2003). Dogs, which were treated postnatally, had improved permanent dentition

81   and normalized lacrimation, as well as improved resistance to airway infections and sweating

82   ability (Casal et al. 2007; Mauldin et al. 2009). Recently, human children were treated

83   prenatally with promising results, too (Schneider et al. 2018). The objective of this study was

84   to characterize the ED phenotype in a litter of shorthaired standard Dachshunds, to determine

85   the most likely mode of inheritance after collection of records of relatives, and to unravel the

86   responsible mutation. Whole-genome sequencing data were used to screen the genome for

87   ED-associated variants and then, we validated the ED-associated variant in the close relatives

88   of the affected Dachshunds, a large panel of different Dachshund breeds and other dog breeds.

89

90   **Materials and Methods**

91   Ethics Statement

92   All animal experiments were performed according to national and international guidelines for

93   animal welfare. All dogs in this study were privately owned and written informed consent of

94   their owners was obtained for this specific study. The Lower Saxony state veterinary office at

95   the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit,

96   Oldenburg, Germany, was the responsible Institutional Animal Care and Use Committee

97   (IACUC) for this study. This particular study was approved by the Niedersächsisches

98   Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES) under the file number

99   33.8-42502-05-18A294. Further dog samples of different breeds used as controls were taken

100   from the biobank of the Institute of Animal Breeding and Genetics at the University of

101   Veterinary Medicine Hanover.

102   Animals

103   Four euthanized male shorthaired standard Dachshund puppies (dogs A, B, C and D) with an

104   ED phenotype were presented at the Institute for Animal Breeding and Genetics of the

105   University of Veterinary Medicine Hanover. The owner reported that the affected puppies

106   developed nasal discharge and respiratory distress, which is why the puppies were euthanized

107   at the age of eight days. The other three puppies were female and showed no signs of a

108   disease. The litter was bred according to the Fédération Cynologique Internationale (FCI)

109   rules within the Deutscher Teckelklub 1888 e.V. (DTK). Pedigrees officially issued by the

110   DTK for the litter with the affected puppies, their parents, grandparents and further relatives

111   were provided by the dog owner.

112   Diagnostic imaging, necropsy and histo-pathology

113   The four affected, eight-day-old male shorthaired standard Dachshund puppies were clinically

114   examined. Three of the four affected puppies (dog B, C and D) were X-rayed to evaluate their

115   dentition. One puppy (dog A) was submitted for necropsy and histo-pathological examination.

116   Tissue samples were fixed in 10% neutral buffered formalin before being embedded in

117   paraffin. For histological examination, 2–3 μm thick sections were cut and stained with

118   hematoxylin and eosin. To compare the results with normal control tissue, formalin-fixed

119   tissue was obtained from a 9-day-old puppy that had to be euthanized due to another reason.

120   DNA and RNA sampling and extraction

121   We took tissue samples of the tails from the four affected puppies and EDTA-blood samples

122   from the dam (dog E) and maternal granddam (dog F). In addition we collected EDTA-blood

123   samples from three female full-siblings of the affected puppies (dog H, I, and J) and two full-

124   siblings of the dam (dog K and L). DNA samples from the maternal great-granddam (dog G)

125   and four paternal half-siblings of the dam (dogs M, N, O, P) were already in our biobank.

126   Genomic DNA extraction was performed using a standard salting out procedure using

127   chloroform. Hair root samples from the dam (dog E) were taken and stored in RNAlater for

128   RNA isolation using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). Reverse

129   transcription reactions from the RNA samples of the dam and a Great Dane reference animal

130   were performed with Invitrogen Super Script IV ReverseTranscriptase (Thermo Fisher

131   Scientific, Waltham, MA, USA).

132   Whole genome sequencing (WGS)

133   We prepared libraries with the NEBNext Ultra II DNA Library Prep Kit for Illumina (New

134   England BioLabs, Ipswich, MA, USA). The libraries of one affected puppy and his dam (dog

135   A and E) were sequenced on an Illumina NextSeq500 (Illumina, San Diego, CA, USA) using

136   the 2x150bp in paired-end mode. Quality control was applied with fastqc 0.11.5 (Andrews

137   2010) and reads were trimmed using PRINSEQ (V 0.20.4) (Schmieder and Edwards 2011).

138   We mapped the reads to the canine reference genome CanFam 3.1 (GCA_000002285.2,

139   Ensembl, www.ensembl.org) using BWA 0.7.13 (Li and Durbin 2009). Sorting, indexing and

140   marking of duplicates was performed using SAMtools 1.3.1 (Li et al. 2009) and Picard tools

141   (http://broadinstitute.github.io/picard/, version 2.9.4). We employed GATK version 4.0

142   (McKenna et al. 2010) using Base Quality Score Recalibration (BQSR), Haplotype Caller and

143   Variant Recalibrator for variant calling. Variant effect estimation was performed on basis of

144   SNPEff v 4.1 (Cingolani et al. 2012) for predictions on CanFam3.1.86 database. For

145   comparison, WGS data from five dogs of the breeds Briard (n=2), German Drahthaar (n=3)

146   and a Chinese Wolf (SRX1137188) were available. We searched the WGS data for

147   hemizygous mutant variants on the X-chromosome and homozygous mutant variants on all

148   autosomes using SAS, version 9.4 (Statistical Analysis System, Cary, NC, USA). Only those

149   variants, which were predicted to have high to moderate effects using the prediction toolbox

150   SNPEff were chosen for further analysis. Additionally we used the Ensembl Variant Effect

151   Predictor (McLaren et al. 2010), with CanFam 3.1 as reference genome, for SIFT predictions

152   (Kumar et al. 2009). We also applied the PolyPhen-2 (Polymorphism Phenotyping v2) tool for

153   variant effect predictions (Adzhubei et al. 2010). The results were compared with a candidate

154   gene list for ectodermal dysplasia (Table S1). Only variants in those genes exclusively found

155   in dog A were considered for further analysis.

156   Validation

157   In total, 12 shorthaired standard Dachshunds closely related with the affected puppies, further

158   37 unrelated shorthaired standard Dachshunds and 128 Dachshunds from other varieties

159   (shorthaired, wirehaired and longhaired in the sizes standard, miniature and rabbit) as well as

160   70 dogs of 34 different breeds from our biobank were used as reference animals. A restriction

161   fragment length polymorphism (RFLP) for mutation detection of the PRJEB27789 variant

162   within *EDA* was developed using the restriction enzyme *Alu*I (New England Biolabs) (Table

163   S2). We designed primers for the polymerase chain reaction (PCR) using Primer3

164   (http://bioinfo.ut.ee/primer3/) (Untergasser et al. 2012) (Table S2). The reaction volume of 20

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165   µl was composed of 2 µl DNA or cDNA, 11.8 µl H_2O, 4.4 µl Q-Solution (Qiagen), 2.2 µl

166   PCR-buffer (Qiagen), 0.5 µl dNTP mix, 0.3 µl (5 u/µl) Taq DNA Polymerase (Qiagen), 0.5 µl

167   forward and 0.5 µl reverse primers. The reaction was performed on a SensoQuest Labcycler

168   thermocycler (SensoQuest, Göttingen, Germany) setting 5 minutes denaturation at 95°C, 34

169   cycles of 94°C for 45 seconds, 61.3°C annealing temperature, 72°C for 45 seconds, a final

170   extension step at 72° for 10 minutes and then 4°C for 5 minutes. The incubation of PCR

171   amplificates (15 µl) with *Alu*I (0.5 µl) was performed with 2.5 µl H_2O and 2 µl Cutsmart

172   buffer (New England Biolabs) at 37°C for 12 hours. Products were separated by gel

173   electrophoresis using 4% agarose gels (Rotigarose, Carl Roth, Karlsruhe, Germany).

174   Genotypes were determined by visual examination under UV illumination (BioDocAnalyze,

175   Biometra, Göttingen, Germany). In addition, we used Sanger sequencing to confirm the *EDA*-

176   mutation (Table S2). PCR and cDNA-PCR products were treated with exonuclease I and

177   FastAP Thermosensitive Alkaline Phosphatase and then sequenced on an ABI 3730xl DNA

178   Analyzer (Eurofins GATC Biotech, Konstanz, Germany). We analyzed the Sanger sequence

179   data with Sequencher 4.8 software (GeneCodes, Ann Arbor, MI, USA). ORFfinder (NCBI,

180   https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict the effects of the mutation on

181   the open reading frame. The wildtype and mutated predicted protein sequences were

182   compared using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al.

183   2011). The position of the protein domains of ectodysplasin were assessed with Ensembl and

184   Superfamily (Gough et al. 2001), Gene3D (Lewis et al. 2018), Prosite_profiles (Sigrist et al.

185   2013) and Pfam (Finn et al. 2016) as domain sources .

186   Data availability

187   The whole-genome sequencing data of the affected puppy and dam were deposited in the

188   NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under the SRA accession

189   number PRJNA309755 (SAMN09435548 and SAMN09435549). The *EDA*-variant is

190   submitted under the accession number PRJEB27789 in the European Variant Archive (EVA).

191   Supplemental material is available at Figshare:

192   EDA_Vasiliadis_Dachshund_Ectodysplasin_de_novo.

193

194   **Results**

195   Clinical presentation

196   All four male standard shorthaired standard Dachshund puppies of the litter showed alopecia

197   in the area of the skullcap, the median area from the base of the tail up to the last ribs, the

198   lower chest and abdomen and the upper inner part of the limbs (Figure 1). The puppies had

199   normal weight at birth. The parents of the affected puppies were phenotypically

200   inconspicuous and officially, FCI-registered standard sized shorthaired Dachshund breeding

201   dogs (Figure 2). Two of the female puppies of the litter (dogs I and J) had mild hypotrichosis

202   of the forehead, ears and lower chest. The female puppies showed no other abnormalities and

203   thrived well.

204   Necropsy and histopathological examination

205   Necropsy and histo-pathological examination was done in one male puppy (dog A). Aside

206   from the alopecia restricted to specific body areas, there were no macroscopic pathological

207   findings. Histologically, there was a focally reduced number and lack of hair follicles in the

208   alopecic areas of the head and back. A medium degree, focal, irregular hyperplasia of the

209   epithelium was present in the skin of the head. There also was a medium degree, focal,

210   subacute, purulent folliculitis as well as a low-grade inflammatory infiltration of both

211   epidermis and dermis. The skin of the footpads missed sweat glands, and the trachea,

212   esophagus and bronchia were devoid of glands. There were no cilia detectable in the trachea,

213   but in the bronchial epithelium. There was no indication of bronchopneumonia (Figure 3).

214   Diagnostic Radiology

215   The full-body radiological images of three affected puppies (dogs B-D) revealed no anomalies

216   of the skeleton. Dental imaging of two of the affected puppies (dog B and D) showed multiple

217   missing premolars and irregularly spaced teeth. However, dog C had a complete dentition and

218   even an additional premolar (Figure 4).

219   Pedigree analysis

220   The pedigree of the litter with the affected puppies was traced back for six generations and

221   phenotype data were supplemented for these dogs. The dam and sire of the affected puppies

222   were unaffected. A litter of maternal half-siblings was inconspicuous as well as all littermates

223   of the dam and granddam and further siblings of all progeny of ancestors. The maternal

224   granddam (dog F) and maternal great-granddam (dog G) had healthy litters from different

225   sires. An autosomal recessive or dominant inheritance was ruled out due to normal parents

226   and ancestors as well as no obvious inbreeding in the six generations. The mode of

227   inheritance suggests an, X-recessive transmission through the dam or the germline of either

228   grandparent (Figure 2).

229   Whole genome sequencing, Sanger sequencing and mutation analysis

230   Whole genome sequencing of dog A revealed a 1-bp deletion in exon 6 within the *EDA* gene

231   (CM000039.3:g.54509504delT, c.458delT). Variants in other ED candidate genes (Table S1)

232   did not fulfill the search criteria. Poly-Phen2 predicted this mutation to be probably damaging

233   with a score of 1.000 (sensitivity: 0.00; specificity: 1.00) using the HumDiv model. Sanger

234   sequencing of DNA-samples of the affected puppy, dam and granddam (dogs A, E and F)

235   confirmed dog A as hemizygous, his dam (dog E) as heterozygous and the granddam (dog F)

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236   as wildtype (Figure 5). A cDNA Sanger sequence of dog E was hemizygous wildtype. This

237   *EDA*-1-bp deletion was predicted to lead downstream to 21 altered amino acids (aa) and a

238   premature stop codon in the canine *EDA* transcripts EDA-201 and EDA-202 (Figure 6). The

239   protein sequence is predicted to lack 85 aa and thus be shortened from 258 to 173 aa

240   (p.H153fsX173). According to the canine reference genome assembly CanFam3.1, the

241   frameshift mutation is localized at amino acid position 153 and therefore in the tumor necrosis

242   factor-like domain or tumor necrosis factor domain of ectodysplasin.

243   Validation

244   PCR-RFLP revealed all four affected littermates as hemizygous mutant. The dam (dog E) was

245   heterozygous and the maternal granddam (dog F) and maternal great-granddam (dog G) were

246   wildtype. Female progeny of the grandsire were wildtype. The mildly hypotrichotic female

247   siblings of the cases (dogs I and J) were heterozygous, and the female littermate with a full

248   coat (dog H) was wildtype. A summary of all available genotypes of the family is given in

249   Table 1. Furthermore, the *EDA*-variant was homozygous wildtype in 37 unrelated shorthaired

250   standard Dachshunds as well as in other Dachshund breeds including shorthaired miniature

251   (n=1), longhaired rabbit (n=6), longhaired standard (n=4), longhaired miniature (n=14),

252   wirehaired rabbit (n=9), wirehaired standard (n=59) and wirehaired miniature (n=35) as well

253   as in the across-breed screen for 34 different dog breeds (Table S3). We found a perfect

254   association of the CM000039.3:g.54509504delT variant with the affected phenotype and

255   confirmed X-linked inheritance.

256

257   **Discussion**

258   In this study, we provide an in-depth description of an XLHED case in a litter of shorthaired

259   standard Dachshunds. The diagnosis XLHED was based on the specific phenotype of the

260   puppies, their pedigree and a mutation analysis using WGS-data. The most obvious signs for

261   XLHED were the partial alopecia with a consistent pattern among all four male cases and the

262   tooth anomalies. This alopecia pattern is identical to those of dogs with confirmed cases of

263   XLHED (Casal et al. 1997; Waluk et al. 2016) and also consistent with many other cases

264   where XLHED is suspected (Moura and Cirio 2004), namely a bald forehead, abdomen and

265   dorsal pelvic area. Secondly, the observed tooth anomalies are typical findings in XLHED

266   (Lewis et al. 2010). Whether the remaining teeth were misshapen could not be determined on

267   the dental X-ray of the eight-day old pups. Subsequently, the evaluation of the permanent

268   dentition is also not applicable. The fact that one of the puppies (dog C) does not exhibit

269   detectable tooth anomalies is not contradictory to the diagnosis of XLHED. In one case, out of

270   17 examined XLHED affected dogs, one had no missing teeth (Lewis et al. 2010), and in

271   another it was stated that there were no tooth abnormalities in the two XLHED-affected, 15

272   month old miniature poodles (Selmanowitz et al. 1970a), suggesting some variance in the

273   severity of affectedness. Another organ system affected in ED are exocrine glands (Cluzeau et

274   al. 2011), which is given in our case. The examined puppy was missing eccrine sweat glands,

275   tracheal and esophageal glands. The absent tracheal glands have led to decreased mucociliary

276   clearance and upper respiratory tract infections, which presented as the nasal discharge and

277   respiratory distress the male puppies were affected with. Though they did not suffer from

278   bronchopneumonia, the signs were severe enough to prompt euthanasia of the affected male

279   puppies.

280   Pedigree analysis revealed that the mode of inheritance is recessive, since the litter was born

281   from unaffected parents. A X-chromosomal inheritance was assumed due to only male

282   affected offspring. Thus, the most likely mode of inheritance is X-recessive with the dam as a

283   heterozygous carrier of the mutation as confirmed through the PRJEB27789 variant. The

284   maternal granddam was homozygous wildtype. A DNA sample could not be obtained from

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285   the grandsire, but his normal phenotype is consistent with a X-chromosomal inheritance. We

286   also did not identify any other offspring of granddam and -sire as carriers of the *EDA*-

287   mutation. That source of the *EDA*-mutation may be most likely in the germline of the

288   granddam, or in an early embryonic stage of the dam (dog E). A germline mutation in the

289   grandsire seems less likely, as first and later offspring generations were normal and ED

290   phenotypes were not reported from this grandsire line. In case of a germline mutation of the

291   granddam, all somatic and germline cells of the dam harbor the mutation. We cannot exclude

292   that the dam is a mosaic of normal and mutated cells, supporting the hypothesis of an early

293   embryonic mutation before germ cells differentiated.

294   With the dam being an X-heterozygous, asymptomatic carrier one would expect that 50% of

295   her male descendants would be affected and 50% of her female offspring carriers. The actual

296   ratio of healthy versus affected male descendants of the dam is 1:4, which is still within

297   statistical limits given the small sample size. Furthermore two of her six daughters (dogs I and

298   J) were identified as carriers. The two carrier females exhibited sparse hair on the

299   frontotemporal area, ears and ventral chest. The dentition of the dam and her carrier daughters

300   at the age of eight weeks was inconspicuous. It is described that carrier females often suffer

301   mild symptoms, too (Kerr et al. 1966; Söderholm and Kaitila 1985; Clarke et al. 1987; Casal

302   et al. 1997). The variance in the severity of signs may be attributed the random inactivation of

303   one X-chromosome in female mammals (lyonization) (Moura and Cirio 2004; Lexner et al.

304   2008; Vogel and Motulsky 2013). Alternatively, if the dam was a mosaic and the amount of

305   cells carrying the mutation would be low (also called a low-level mosaicism), this would lead

306   to the same phenotype as a skewed X-inactivation to the healthy side. Low-level mosaicism in

307   the dog is a rare condition, but may be underdiagnosed (Switonski et al. 2003).

308   For the mutation detection in the WGS data, the filtering criteria left only *EDA* as a candidate

309   gene. This was expected, since the other most common ED candidate genes do not match our

310   observed phenotype and mode of inheritance. *EDAR* and *EDARADD* mutations would cause a

311   clinically indistinguishable phenotype, but are inherited as autosomal, and *WNT10A* also

312   causes a distinct phenotype with nail dystrophy (Cluzeau et al. 2011). Mutations in further ED

313   candidate genes *TNF receptor associated factor 6 (TRAF6), Inhibitor Of Nuclear Factor*

314   *Kappa B Kinase Subunit Gamma (IKBKG,* formerly *NEMO), NFκB inhibitor α (NFKBIA)* and

315   *NFκBs* also cause immunodeficiency (Cui and Schlessinger 2006) and variants with effects on

316   protein function were not identified in these candidates. Subsequently the

317   CM000039.3:g.54509504delT variant was the only one that met all conditions and was

318   validated.

319   The mutation c.458delT causes a frameshift, leading to altered aa and a premature stop codon

320   and thus shortening of all canine ectodysplasin-A isoforms. The mutation was predicted to

321   affect the tumor-necrosis-factor domain of the protein, thus probably rendering this domain

322   useless (Schneider et al. 2001). Depiction of the mutated mRNA by Sanger sequencing a

323   cDNA-PCR product of the heterozygous dog E was attempted, but apparently, only the

324   mRNA from the intact chromosome was sequenced, so the Sanger sequence came out as

325   (hemizygous) wildtype. We therefore speculate that in case of a mosaicism, it is possible that

326   the hair collection site (the tail) consisted only of normal cells. Another possibility is that the

327   mutant mRNA is produced, but quickly detected as faulty by the organism and therefore

328   degraded. It is possible that the small fragments of the degraded mRNA showed up as mild

329   interference in the chromatogram of the Sanger sequence. The degradation of mRNA with

330   premature stop codons is a well-known phenomenon, called nonsense-mediated decay

331   (Wagner and Lykke-Andersen 2002). The degradation can be initiated by the removal of the

332   mRNA poly-A tail, which provides access for degradative enzymes to the body of the mRNA

333   (Wilusz et al. 2001). If the dam´s X-inactivation was skewed to the healthy side, so that more

334   of the mutant than of the healthy chromosome was inactivated, this would leave a large

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335   enough amount of functional ectodysplasin for her to not develop ED signs. Alternatively, if

336   the mutant *EDA* was transcribed, the protein would be nonfunctional because of the damaged

337   TNF-domain.

338   The TNF-domain is crucial to the function of ectodysplasin-A (Schneider et al. 2001). The

339   TNF-domain of this type II membrane protein is located extracellular (Ezer et al. 1999). From

340   there the TNF-domain is cleaved to produce a soluble ligand that forms trimers and interacts

341   with its respective receptors (Elomaa et al. 2001; Chen et al. 2001). One isoform of

342   ectodysplasin-A, EDA-A2, is specific to the receptor EDA2R (Yan et al. 2000). This pathway

343   can activate NF- κB in vitro (Yan et al. 2000; Sinha et al. 2002), but not in-vivo (Gaide and

344   Schneider 2003; Mustonen et al. 2003). Furthermore, the role of EDA2R signaling seems

345   expendable in skin development, as *EDA2R* knockout mice do not show an ED phenotype

346   (Newton et al. 2004). EDA-A1, however, is well known for binding to its receptor EDAR

347   (Yan et al. 2000). EDAR in turn binds to its cytoplasmic signaling adaptor EDARADD

348   (Headon et al. 2001), which via TRAF6 activates the IκB kinase (IKK) complex (Morlon et

349   al. 2005). From there on, apparently the IKBKG/IkBα-dependent NF-κB cascade is initiated

350   (Cui and Schlessinger 2006; Weih and Caamaño 2003). The NF-κB pathway is crucial for the

351   development of skin appendages. Thus it is plausible, that a mutation in the TNF domain of

352   ectodysplasin-A, leads to impaired binding of both isoforms to their receptors (Schneider et

353   al. 2001), subsequently leading to a disrupted EDA/NF-κB signaling pathway and manifesting

354   itself in XLHED. In conclusion, this study demonstrates the first XLHED cases in the

355   shorthaired standard Dachshund, most likely due to an *EDA* germline mutation in the dam or

356   maternal granddam. The PRJEB27789 variant is predicted to cause a nonfunctional protein

357   due to a damage of the TNF-domain needed for the *EDA* signaling pathway. Variance in

358   affectedness of carrier females was obvious and possibly attributable to a skewed X-

359   inactivation, or, in case of the dam, to low-level mosaicism.

16

360

Acknowledgements

368

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558

559 **Figures and Tables**

560   **Figure 1** Phenotypic appearance of a male affected shorthaired standard Dachshund (dog A).

561   The characteristic alopecia pattern is easily recognizable. (A) The forehead and the chest with

562   the well demarked hypotrichosis. (B) The whole underbelly is hairless, too. Note that the nails

563   and footpads are developed normally. (C) The alopecia also extends in a dorsal stripe from the

564   base of the tail up to the last ribs. The three other affected male puppies shared the exact

565   phenotype. Three of the puppies were black and tan and one was brown.

566   **Figure 2** Pedigree of the affected shorthaired standard Dachshund family. Family members,

567   which are mentioned in this study, are marked with the letters A to P. Also given are the

568   genotypes for every dog we were able to get DNA samples from. A square symbol means a

569   male individual, and a circle a female. Filled out symbols mark the XLHED-affected

570   individuals.

571   **Figure 3** Histopathology of the eight days old Dachshund puppy (right panels) compared to a

572   9-days-old control puppy (left panels). In normal skin, numerous hair follicles are visible (A),

573   while in alopecic areas the hair follicle number is highly reduced with a segmental absence

574   (B). Numerous eccrine sweat glands in the fat pad can be detected in the control footpad (C),

575   whereas in the examined Dachshund puppy no glandular structures are recognizable (D).

576   Individual bronchial glands can often be observed next to cartilage structures (E), whereas in

577   the examined Dachshund puppy no glandular structures were detected in the bronchi (F). In

578   the submucosa of the trachea (G) and esophagus (I), numerous nests of glands are usually

579   recognizable, while they are completely absent in the examined Dachshund puppy (H, J).

580   (Hematoxylin and eosin stains; Bar in A, B, C, D: 200µm; Bar in E and F: 50 µm; Bar in G,

581   H, I, J: 100 µm).

582   **Figure 4** Dental imaging for dogs B, C and D. Depicted are the mandibular (right) and

583   maxillar (left) X-rays of three of the affected male puppies. A healthy puppy should have the

584   following developing teeth in the upper and lower jaw at the age of eight days: six incisors,

25

585 two canines, four premolars and two molars. In the upper jaw of dog B (A1), two premolars

586 are missing, the lower jaw (A2) has a full bite but with irregularly spaced teeth. Dog C has a

587 full bite in both jaws and additional premolar in the lower jaw (B2). Dog D has two missing

588 premolars in the upper jaw (C1) and three missing premolars in the lower jaw (C2).

589 **Figure 5** Sanger sequencing chromatograms of dogs A, E and F. The granddam (dog F) was

590 homozygous wildtype. At position *EDA*:c.458 the wildtype allele T is present. One of the

591 affected male puppies shows at position *EDA*:c.458 deletion of the allele T (dog A). His dam

592 (dog E) is heterozygous resulting in a shift of the sequence with the PRJEB27789 variant.

593 **Figure 6** Given are the known ectodysplasin-A-protein isoforms of the canine *EDA* gene and

594 the predicted effect of the *EDA*:c.458delT frameshift mutation on their proteins. First, we

595 compared the various wildtype protein sequences as stated by the databases Ensembl and

596 NCBI (A). Then we present the respective predicted mutant protein sequences (B). The

597 frameshift mutation was predicted to lead to 21 altered amino acids and a premature stop

598 codon in the *EDA* transcripts EDA-201 and EDA-202 (Ensembl). The produced proteins

599 therefore would lack 85 amino acids. The database NCBI (https://www.ncbi.nlm.nih.gov/)

600 lists another five ectodysplasin-A isoforms, which would also be shortened by 85 to 30 amino

601 acids. The stated protein sequences of both databases differ. EDA-201 and EDA-202 have the

602 same length of 258 amino acids, but differ on 10 amino acids. Ectodysplasin-A as stated by

603 NCBI has a sequence identical to EDA-201 but is 128 amino acids longer, as the predicted

604 transcription starts earlier. The other isoforms of ectodysplasin-A are one to five amino acids

605 shorter and with a few non-matching amino acids. According to the canine reference genome

606 assembly CanFam3.1, the frameshift mutation is localized at amino acid position 153 (281

607 resp. 278 for the NCBI-isoforms) and therefore in the tumor necrosis factor-like domain or

608 tumor necrosis factor domain of ectodysplasin. In each case, the mutation leads to an altered

609   and shortened protein sequence, thus rendering the tumor-necrosis-factor-domain of the

610   ectodysplasin-A isoforms useless.

611   **Table 1** Genotyping results for the CM000039.3:g.54509504delT variant of the affected

612   Dachshund family and reference dogs. The Dachshund breed, the number of individuals per

613   breed, and the respective sex and genotype are stated. For the affected dachshund family, the

614   predicted genotype is also stated, when it could be deducted from phenotype and/or mode of
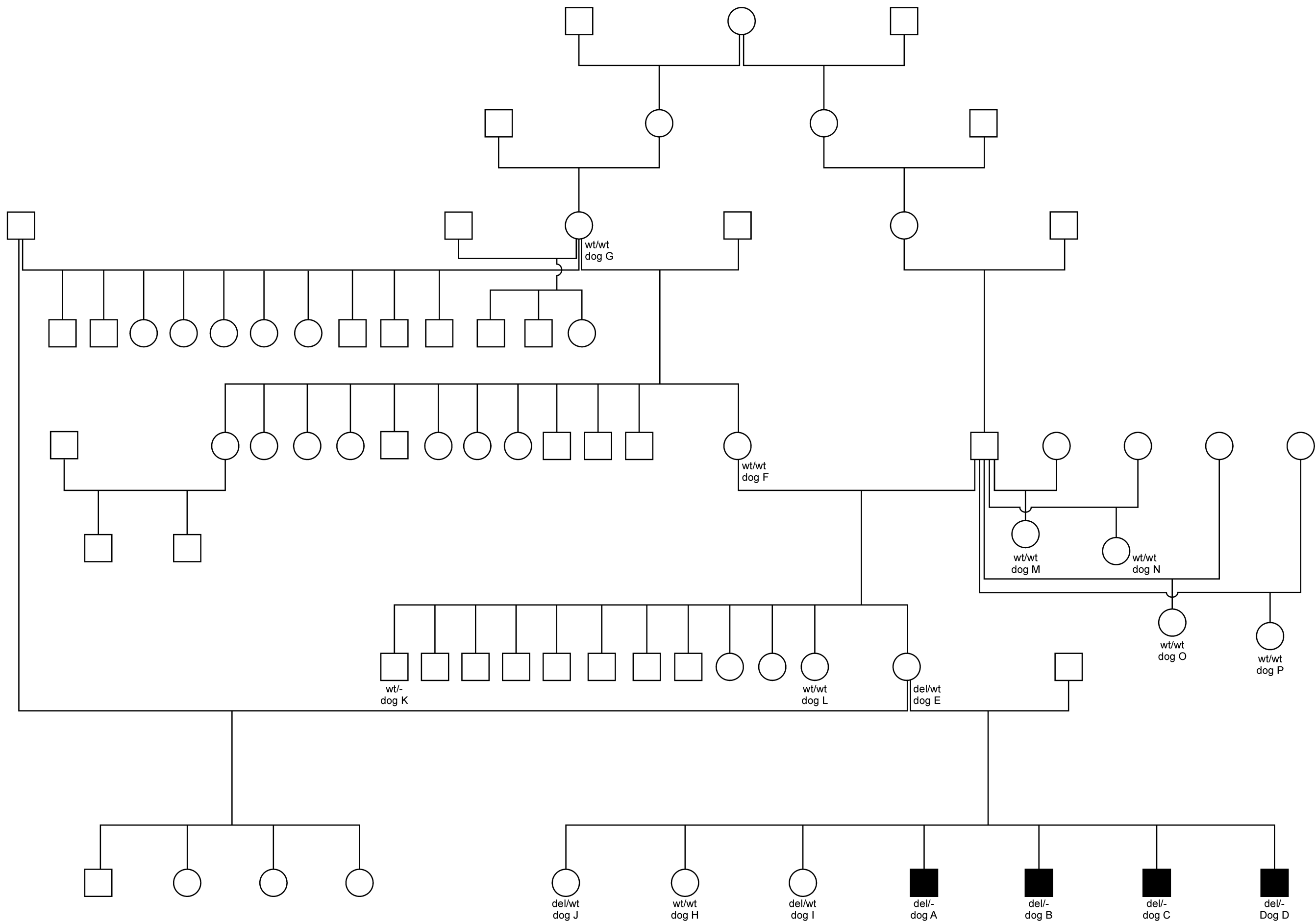
615   inheritance.

616
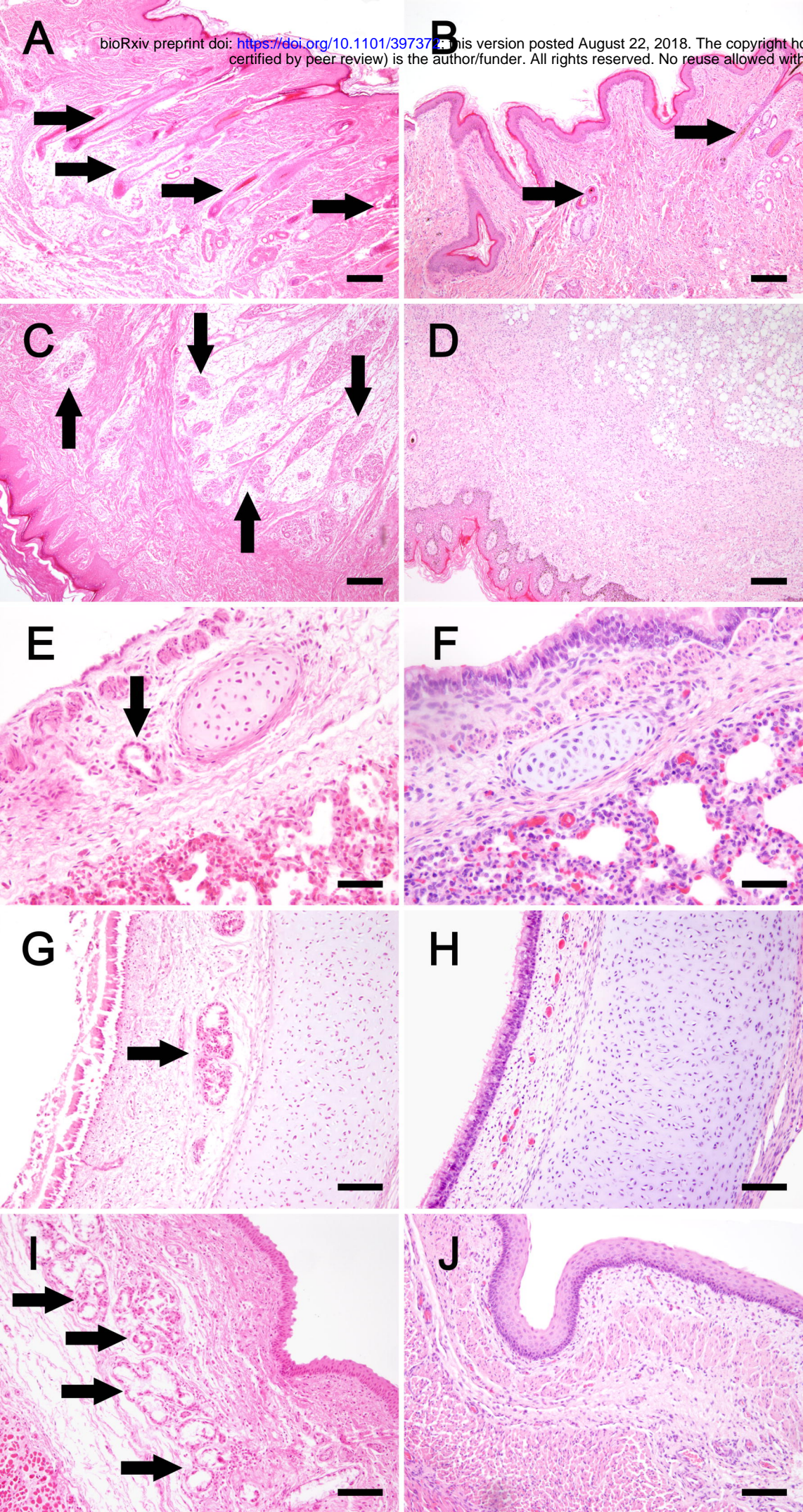
617

618   **Supplementary Files**

619   **Table S1** Candidate gene list analysis. Genes potentially involved in ectodermal dysplasia

620   were identified using NCBI Gene database. The canine chromosome position, genes, gene ID
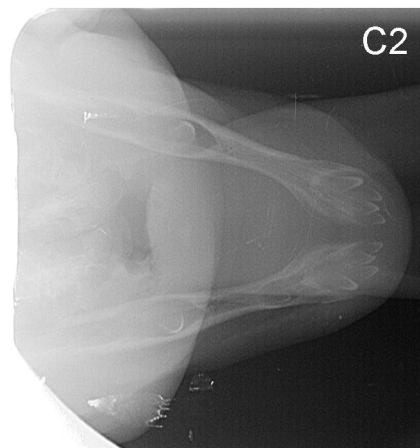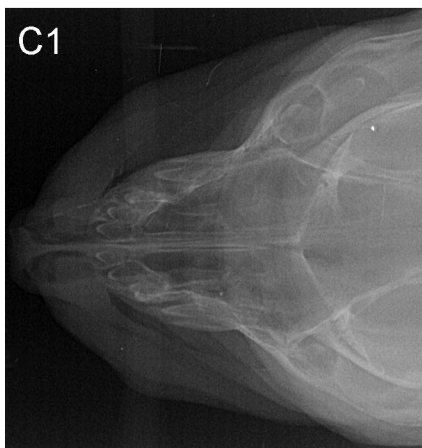
621   and name are shown.

622   **Table S2** Primer sequences used for validation of the mutation detected by whole genome

623   and Sanger sequencing. The variant CM000039.3:g.54509504delT, c.458delT was validated

624   by the use of restriction fragment length polymorphism (RFLP). Primer pairs, amplicon size

625   (AS) in base pairs (bp), annealing temperature (AT), restriction enzyme and incubation

626   temperature (IT) are given. Also depicted are the three primers used for cDNA Sanger

627   sequencing.

628   **Table S3** Genotyping results for the CM000039.3:g.54509504delT variant of reference dogs

629   of other breeds. The dog breed, the number of individuals per breed, and the respective sex

630   and genotype are stated. 70 dogs of 34 breeds were tested in total.

wt/wt
dog G

wt/wt
dog F

wt/wt
dog M

wt/wt
dog N

wt/wt
dog O

wt/wt
dog P

wt/-
dog K

wt/wt
dog L

del/wt
dog E

del/wt
dog J

wt/wt
dog H

del/wt
dog I

del/-
dog A

del/-
dog B

del/-
dog C

del/-
Dog D

female wildtype (dog F)

C C A A G G T G T T T A A G C T A C A T C C C C G C
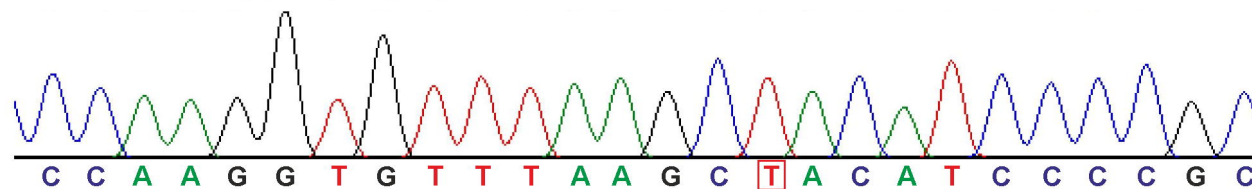
heterozygous dam (dog E)   c.458delT

C C C A A G G T G T T T A A G C T A C A T C C C C G C
A C A T C C C C G C A
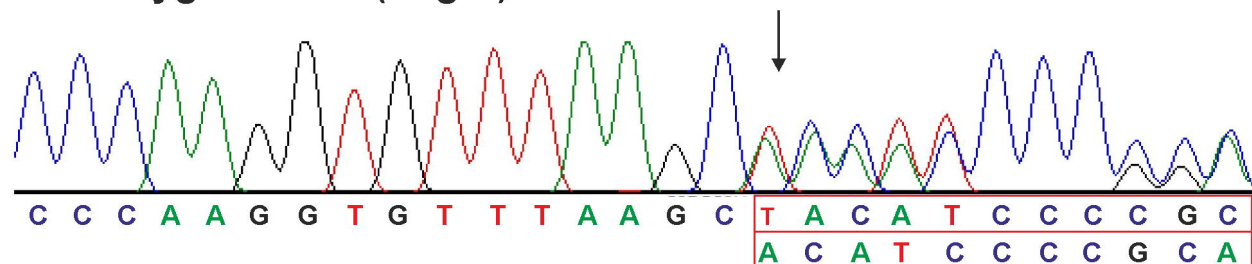
hemizygous male puppy (dog A)   c.458delT

C C C A A G G T G T T T A A G C C A C A T C C C C G C
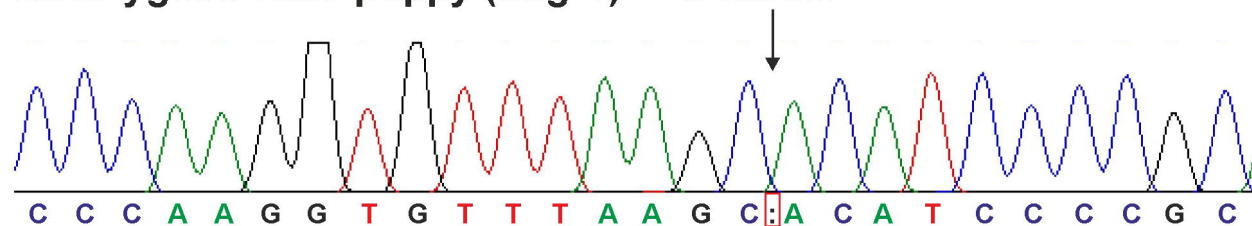
A

ENSEMBL_EDA_201_WILDTYPE p.L153

113 258

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ENSEMBL_EDA_202_WILDTYPE p.L153

113 258

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NCBI_ECTODYSPLASIN-A_WILDTYPE p.L281

241 386

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NCBI_ISOFORMX1_WILDTYPE p.L281

241 385

...NQPAVVHLQGQGSAIQVKNDLSGGVLNDWSRITMNPKVFK L HPRSGELEVLVDGTYFIYS...EPTSV*

NCBI_ISOFORMX2_WILDTYPE p.L281

241 384

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NCBI_ISOFORMX3_WILDTYPE p.L278

241 383

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NCBI_ISOFORMX4_WILDTYPE p.L278

241 381

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B

ENSEMBL_EDA_201_MUTANT p.H153fsX173

113 173

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ENSEMBL_EDA_202_MUTANT p.H153fsX173

113 173

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NCBI_ECTODYSPLASIN-A_MUTANT p.H281fsX301

241 301

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NCBI_ISOFORMX1_MUTANT p.H281fsX301

241 301

...NQPAVVHLQGQGSAIQVKNDLSGGVLNDWSRITMNPKVFK H IPAAGSWRYWWTAPTSSIVR*

NCBI_ISOFORMX2_MUTANT p.H281fsX354

241 354

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NCBI_ISOFORMX3_MUTANT p.H278fsX298

241 298

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NCBI_ISOFORMX4_MUTANT p.H278fsX351

241 351

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