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## 5 **The First Case of Genetically Confirmed Monozygotic Twinning in the Dog**

6

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15

### 16 **Contents**

17 Monozygotic twinning has not previously been genetically confirmed in the dog. This case report  
18 describes the finding of two viable male monozygotic foetuses within one placental site during  
19 caesarean section. Their umbilical cords attached to a single placenta. Genetic profiling using a  
20 total of 38 microsatellite markers, as well as amelogenin and SRY for sex determination, revealed  
21 identical DNA profiles, whether derived from blood or tissue (buccal swabs) samples. To the best  
22 of our knowledge, this is the first report of monozygotic twinning in the dog confirmed using DNA  
23 profiling.

24

25 **Keywords:** canine, genetic, monozygous, monochorionic

26

27 **Abridged title:** Monozygotic twinning in the dog

28

29 **Introduction**

30 Monozygotic twinning has been reported in the horse (Govaere et al. 2009), cow (Del Rio et al.  
31 2006) and pig (Bjerre et al. 2009), and is presumed to be extremely rare in the mouse (McLaren et  
32 al. 1994) and rabbit (Bomse-Helmreich and Papiernik-Berkhauer 1976). In contrast, the nine-  
33 banded armadillo (*Dasypus novemcinctus*), and possibly other species of the genus *Dasypus*  
34 (Loughry et al. 2015), consistently produces genetically identical quadruplets through binary  
35 fission events, lending itself to the study of the mechanism behind monozygotic twinning which is  
36 currently poorly understood (Blickstein and Keith 2007). In humans, spontaneous monozygotic  
37 twinning occurs at the rate of approximately one in 330 livebirths (Hall 2003).

38

39 Monozygotic twinning has not previously been genetically confirmed in the dog. Duke (1946)  
40 described two dog embryos within one placental site. A presumptive diagnosis of monozygotic  
41 twinning was based on the finding of a single chorion and yolk sac; each embryo having possessed  
42 its own amnion. The embryos had not yet undergone sexual differentiation.

43

44 Conjoined twinning has been reported rarely in the dog (Mainland 1929, Mazzullo et al. 2007,  
45 Nottidge et al. 2007, Paquet et al. 2011, House et al. 2012). Furthermore, the sharing of a single  
46 placental site by dizygous dog fetuses has been described rarely (Urhausen et al. 2013, Joonè et  
47 al. 2015).

48

49 **Case report**

50 A four year old, multiparous Irish wolfhound bitch was presented to a veterinary facility during  
51 second-stage labour. The bitch had had one previous litter of 10 puppies, the last five of which  
52 were delivered by emergency caesarean section. At presentation, the owner reported that the bitch  
53 had been showing tenesmus for two hours without the expulsion of a foetus. No vulvar discharge  
54 was present. Due to the extended period of unproductive tenesmus, a caesarean section was  
55 performed.

56

57 Upon exposure of the uterus, the surgeon noticed a bulge near the base of one of the uterine horns,  
58 approximately the length of a single foetus. Via a longitudinal incision into the body of the uterus,  
59 one foetus (twin A) was delivered from this section of uterus. A second foetus (twin B) was  
60 immediately noticed within the same chorionic bag. Without rupturing either pup's umbilical cord,  
61 the second pup and the placenta were delivered from the uterus. Both pups' umbilical cords, which  
62 were similar in length to the rest of the litter's, attached to the same placenta (Figure 1). Five more  
63 live, normal puppies were delivered with different placentae.

64

65 At two weeks of age, blood samples from twins A and B were collected via jugular venipuncture  
66 into EDTA vacutainer tubes for genetic analysis. At six weeks of age, blood was similarly  
67 collected from the five non-twin members of the litter. In addition, buccal swabs were collected  
68 from twins A and B by twirling a dry swab against the inside of the cheeks for at least 15 s.

69

70 Genetic analyses were performed by the Veterinary Genetics Laboratory (VGL; University of  
71 Pretoria, South Africa). Extraction of DNA from whole blood and buccal swabs was performed  
72 using the Prepfiler™ Forensic DNA Extraction Kit (Applied Biosystems, Foster City, USA) and  
73 the Genra Puregene Tissue Kit (Qiagen, Valencia, USA), respectively, according to the  
74 manufacturers' instructions. Genetic profiles were generated using a panel of 24 short tandem  
75 repeat (STR) microsatellite markers and the amelogenin marker for sex determination. Twenty-one  
76 of these markers and the amelogenin marker are recommended by the International Society of  
77 Animal Genetics (ISAG; <http://www.isag.us/Docs/consignmentforms/2005ISAGPanelDOG.pdf>,  
78 accessed 3 June 2016) for dog parentage verification. A further three markers augmented the panel.  
79 Primer design, chromosome position, number of alleles and fragment size ranges have been  
80 described previously (Pedersen et al. 2012). Polymerase chain reaction (PCR) for this panel  
81 consisted of an initial activation step of 10 min at 95°C, followed by 30 cycles of 95°C for 60 s,  
82 56°C for 30 s and 72°C for 60 s. A further panel consisting of 14 tetranucleotide STR  
83 microsatellite markers and a marker for the SRY gene was also utilised. Primer design and PCR  
84 conditions were as previously described (Wictum et al. 2013). Polymerase chain reaction was

85 performed using a 9800 Fast Thermal Cycler (Life Technologies, Johannesburg, South Africa),  
86 followed by capillary electrophoresis by an ABI 3500 XL Genetic Analyser (Life Technologies).  
87 Fragment sizes for each marker were evaluated using the software program STRand Version 2.4.49  
88 (University of California, Davis, USA; Toonen and Hughes 2001).

89

## 90 **Results**

91 Twins A and B were phenotypically normal males. At birth, twins A and B weighed significantly  
92 less ( $t$  test;  $P < 0.001$ ) than their five littermates, however this difference had lost statistical  
93 significance by the age of 6 weeks ( $P = 0.32$ ; Table 1). Although remarkably similar in physical  
94 appearance, they showed slight differences in terms of the size and shape of white markings on the  
95 chest, lower legs and the tip of the tail (Figure 2).

96

97 The DNA profile derived from whole blood matched that derived from tissue (buccal swabs) for  
98 each twin, A and B. Further, the DNA profiles of twins A and B were identical at all 40 genetic  
99 markers. The DNA profiles of all seven littermates are shown in Table 2. Excluding the  
100 comparison between twins A and B, at which no loci were different, the genetic profiles of the  
101 littermates differed at a median of 14 loci (range 8 to 20), excluding amelogenin and SRY.

102

## 103 **Discussion**

104 The current study describes the finding of viable, monochorionic, monozygotic littermates in the  
105 dog. In polytocous species such as the dog, all littermates are essentially twins, triplets, quadruplets  
106 and so on, depending on the size of the litter. Thus the term “twin”, herein used to refer to the  
107 monozygotic “twins” only, should be used with care in these species.

108

109 This study made use of 38 STR microsatellite markers as well as markers for amelogenin and  
110 SRY, exceeding the eight and twelve microsatellite markers previously used to determine  
111 monozygosity in bovine and equine twins, respectively (Del Rio et al. 2006, Govaere et al. 2009).  
112 All 40 loci showed absolute identity between twins A and B. This, together with the finding of

113 both foetuses within one placental site during caesarean section, provides strong evidence for  
114 monozygosity.

115

116 The profiling of DNA derived from buccal swabs, essentially tissue samples, ruled out the  
117 possibility of blood chimaerism as an explanation for identical genetic profiles derived from two  
118 blood samples. In a previous report of blood chimaerism in two dog foetuses, the finding of more  
119 than two alleles at multiple loci on DNA profiles derived from blood samples alerted workers to  
120 the possibility of cross-foetus mixing of the blood supplies *in utero*. Subsequent profiling of tissue  
121 samples provided dissimilar genetic profiles, with no more than two alleles present per marker  
122 (Joonè et al. 2015). In the current study, the blood- and tissue-derived profiles for each individual  
123 were identical. In addition, no loci in either the blood- or tissue-derived profiles showed more than  
124 two alleles.

125

126 In human monozygotic twins, examination of the foetal membranes has been suggested to indicate  
127 the timing of the twinning event (Hall 2003). Due to time constraints involved in the delivery of  
128 living puppies, the surgeon was unable to assess whether twins A and B were within a single  
129 amnion at delivery—precluding any useful estimation of the timing of embryonic fission in the  
130 current study.

131

132 Conjoined monozygotic twins are believed to arise from the incomplete splitting of an embryo  
133 after formation of the primitive streak has begun. In humans, one in 400 monozygotic twins are  
134 reportedly conjoined (Hall 2003). According to Gupta et al. (2001), one to 2 percent of human  
135 conjoined twins are asymmetric (referred to as heteropagus). Logrono et al. (1997) found that, in a  
136 case of human heteropagus conjoined twinning, the parasite and autosite were dizygous;  
137 presumably resulting from the fusion of two conceptuses. Thus, conjoined twins may be  
138 monozygotic due to fission, but need not be. Conjoined twinning has been reported rarely in the  
139 dog (Mainland 1929, Mazzullo et al. 2007, Nottidge et al. 2007, Paquet et al. 2011, House et al.  
140 2012) and no DNA analyses were performed in the described cases. Nevertheless, the small

141 number of cases of conjoined twins in dogs reported in the literature, most of which describe  
142 symmetrical conjoined twinning involving a degree of posterior duplication, suggest that  
143 monozygotic twinning in the dog is rare or that splitting events giving rise to conjoined  
144 monozygotic twins are rare in this species.

145

146 The monozygotic puppies described in the current study were viable and vigorous at birth, despite  
147 having shared a placental site. This finding contrasts to previous reports of two dog foetuses within  
148 one placental site, where death of the foetuses was detected 52 days after ovulation (Urhausen et al.  
149 2013) and at term (Joonè et al. 2015). Therefore, the sharing of a placental site may not be  
150 incompatible with survival to term and beyond, as suggested previously (Joonè et al. 2015).

151

152 Of interest in this case report is the slight differences observed between the monozygotic twins in  
153 the white markings on the paws, the tip of the tail and the chest. Similar findings have been  
154 described in monozygotic twin horses and cattle (Ozil 1983, Allen and Pashen 1984), as well as in  
155 cloned dogs (Hosseini et al. 2009). Woolf (1995) concluded that stochastic events during  
156 development resulted in different white colour markings among the legs of horses in spite of the  
157 legs having had the same genotype and having developed in the same environment. We do not  
158 know whether such stochastic events caused the phenotypic differences between the twins of the  
159 current case. Wong et al. (2005) concluded that variation in phenotype due to epigenetic  
160 differences is smaller in monozygotic twins than in isogenic dizygotic twins because monozygotic  
161 twins share an oocyte and, thereby, have a larger shared epigenomic background than isogenic  
162 dizygotic twins. Wong *et al.*, nevertheless, concluded that epigenetic differences between  
163 monozygotic twins do occur. It is not known whether epigenetic differences would explain the  
164 colour differences between the monozygotic twins in the current case. Given that dog littermates  
165 often look strikingly similar, slight phenotypic differences between monozygotic dogs would  
166 effectively mask their monozygosity, and may have played a role in this phenomenon having gone  
167 undetected until now.

168

169 For genetic identification and parentage analysis purposes, this study shows that dogs with  
170 identical genetic profiles, although likely rare, do exist. Bitches may have more conceptuses in the  
171 litter than they have corpora lutea (Andersen and Simpson 1973, Bysted et al. 2001). One cause for  
172 this may be multiovular follicles (Telfer and Gosden 1987, Reynaud et al. 2009) from which more  
173 than one oocyte may be fertilised. The current case confirms that monozygotic twins is another  
174 possible reason for finding more conceptuses than corpora lutea in bitches.

175

## 176 **Conclusion**

177 This report describes the finding of monozygotic twinning in the dog, confirmed by DNA  
178 profiling. To the best of our knowledge, this is the first report of confirmed monozygotic twinning  
179 in the dog.

180

181

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184 cost of the DNA analysis.

185

## 186 **Author contributions**

187 CJ Joonè wrote the manuscript. KGM De Cramer and JO Nöthling assisted in drafting manuscript  
188 up to the final drafts. KGM De Cramer performed data collection.

189

## 190 **Conflicts of interest**

191 Conflicts of interest: none

192

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269

270 **Figure captions**

271 Fig. 1. Monozygotic twins A and B photographed after delivery while still connected to the single  
 272 placenta via their umbilical cords.

273 Fig. 2. Monozygotic twins A and B photographed with their dam at six weeks of age. Note the  
 274 differences in the white markings on the chest and paws.

275

276

277 **Table 1. Weights of twins A and B and their littermates, at birth and at the age of six weeks.**

<b>Puppy</b>	<b>Weight (g) at birth</b>	<b>Weight (kg) at six weeks of age</b>
Brindle male	755	6.0
Brindle female	743	5.9
Light female	723	5.5
Dark brindle male	790	6.9
Dark brindle female	777	6.1
Twin A	450	5.5
Twin B	530	5.8
Mean (Twins A and B)	490 <sup>a</sup>	5.7 <sup>a</sup>
Mean (Non twins)	758 <sup>b</sup>	6.1 <sup>a</sup>

Means bearing different superscripts within a column differ significantly (P < 0.05)

278

279

280 **Table 2. Genetic profiles derived from seven littermates including monozygotic twins A and**  
 281 **B**

<b>Locus</b>	<b>Light female</b>	<b>Brindle male</b>	<b>Brindle female</b>	<b>Dark brindle male</b>	<b>Dark brindle female</b>	<b>Twin A*</b>	<b>Twin B*</b>
AHT121	104	96,104	96,104	96,104	96,104	96,104	96,104
AHT137	131	131	131	–	131	131	131
AHT <sub>h</sub> 130	129	129	129	129	129	129	129
AHT <sub>h</sub> 171	219	219	219	219	219	219	219
AHT <sub>h</sub> 260	244	244	244	–	244	244	244
AHT <sub>k</sub> 211	91	91	91	91	91	91	91
AHT <sub>k</sub> 253	288,292	288,292	288,292	288,292	288,292	288	288
AMEL	XX	XY	XX	–	XX	XY	XY
CXX279	118,122	122,124	122	122	122,124	122	122
FH2001	136,148	148	136,148	136,148	136,148	148	148
FH2054	156,172	156,172	156,172	156,172	172	172	172

FH2328	200	200,204	200	200,204	200	200	200
FH2848	–	–	–	–	–	238,242	238,242
INRA21	99,101	99,101	99,101	99,101	99,101	99,101	99,101
INU005	124,132	124,132	124,132	132	124,132	132	132
INU030	144,152	144,152	144	–	144,152	144,152	144,152
INU055	214,218	214,220	214,220	–	214,220	218,220	218,220
LEI004	95	95	95	–	95	95	95
REN105LO <sub>2</sub>	231,241	231	231,241	–	231,241	231	231
REN162C04	202	202	202	202	202	202	202
REN169D01	216	216	216	–	216	216	216
REN169O18	164,168	162,164	164,168	164,168	162,164	164,168	164,168
REN247M2 <sub>2</sub>	268,278	268,278	278	–	268,278	278	278
REN54P11	228,236	228,240	228,236	228,236	228,240	228,240	228,240
REN64E19	147,153	145,149	145,149	145,149	149,153	145,147	145,147
SRY	–	Y	–	Y	–	Y	Y
VGL0760	21.1	21.1	21.1	21.1	21.1	21.1	21.1
VGL0910	17.1	17.1	17.1	17.1	17.1	17.1	17.1
VGL1063	17.3,18. <sub>2</sub>	13,18.3	13,18.3	13,18.3	13,18.3	13,17.3	13,17.3
VGL1165	29,30	16,30	29,30	29,30	29,30	16,30	16,30
VGL1541	18	17,18	17	17,18	18	17	17
VGL1828	20	20,21	20	20	20,21	20,21	20,21
VGL2009	9	9,15	9,15	9	9	15	15
VGL2136	15	15,16	15,16	15	15	15,16	15,16
VGL2409	19	18,19	19	18,19	19	18,19	18,19
VGL2918	21,22	22,24	21,23	23,24	21,22	21,23	21,23
VGL3008	12	12	12	12	12	12	12
VGL3112	14	13	13	13	13	14	14
VGL3235	13,16	13,16	12,13	12,13	13,16	12,13	12,13
VGL3438	14	14,17	14,17	14	14	14,17	14,17

282 Data shows DNA fragment lengths, in base pairs, produced for 40 genetic markers including  
283 amelogenin and SRY for sex determination. \*The profiles generated from blood and tissue samples  
284 for twins A and B were identical, therefore no distinction is made between blood or tissue samples  
285 for these individuals. –, indicates a marker that failed to amplify.







