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SHORT COMMUNICATION

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# The first case of genetically confirmed monozygotic twinning in the dog

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**Contents**

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

## 1 | INTRODUCTION

Monozygotic twinning has been reported in the horse (Govaere et al., 2009), cow (Del Rio, Kirkpatrick, & Fricke, 2006) and pig (Bjerre, Thorup, Jørgensen, Vejlsted, & Fredholm, 2009), and is presumed to be extremely rare in the mouse (McLaren, Molland, & Signer, 1994) and rabbit (Bomsel-Helmreich & Papiernik-Berkhauer, 1976). In contrast, the nine-banded armadillo (*Dasypus novemcinctus*), and possibly other species of the genus *Dasypus* (Loughry, Superina, McDonough, & Abba, 2015), consistently produces genetically identical quadruplets through binary fission events, lending itself to the study of the mechanism behind monozygotic twinning which is currently poorly understood (Blickstein & Keith, 2007). In humans, spontaneous monozygotic twinning occurs at the rate of approximately one in 330 live births (Hall, 2003).

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

Conjoined twinning has been reported rarely in the dog (House, Barrand, & Cornillie, 2012; Mainland, 1929; Mazzullo, Monteverde, Macri, Partanna, & Caracappa, 2007; Nottidge, Omobowale, Olopade, Oladiran, & Ajala, 2007; Paquet, El-Warrak, Laguë, & Boerboom, 2011). Furthermore, the sharing of a single placental site by dizygous dog foetuses has been described rarely (Joonè, De Cramer, & Nöthling, 2015; Urhausen et al., 2013).

## 2 | CASE REPORT

A 4-year-old, multiparous Irish wolfhound bitch was presented to a veterinary facility during second-stage labour. The bitch had had one previous litter of 10 puppies, the last five of which were delivered by emergency caesarean section. At presentation, the owner reported that the bitch had been showing tenesmus for two hours without the expulsion of a foetus. No vulvar discharge was present. Due to the extended period of unproductive tenesmus, a caesarean section was performed.

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

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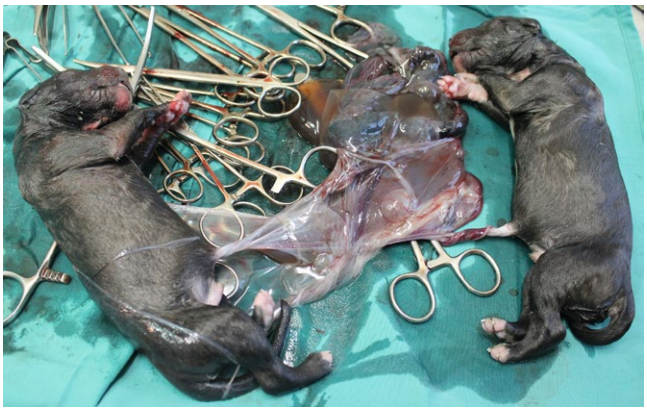

**FIGURE 1** Monozygotic twins A and B photographed after delivery while still connected to the single placenta via their umbilical cords

At 2 weeks of age, blood samples from twins A and B were collected via jugular venipuncture into EDTA vacutainer tubes for genetic analysis. At 6 weeks of age, blood was similarly collected from the five non-twin members of the litter. In addition, buccal swabs were collected from twins A and B by twirling a dry swab against the inside of the cheeks for at least 15 s.

Genetic analyses were performed by the Veterinary Genetics Laboratory (VGL; University of Pretoria, South Africa). Extraction of DNA from whole blood and buccal swabs was performed using the Prepfiler™ Forensic DNA Extraction Kit (Applied Biosystems, Foster City, CA, USA) and the Gentra Puregene Tissue Kit (Qiagen, Valencia, CA, USA), respectively, according to the manufacturers' instructions. Genetic profiles were generated using a panel of 24 short tandem repeat (STR) microsatellite markers and the amelogenin marker for sex determination. Twenty-one of these markers and the amelogenin marker are recommended by the International Society of Animal Genetics (http://www.isag.us/Docs/consignmentforms/2005ISAGPanelDOG.pdf, accessed 3 June 2016) for dog parentage verification. Primer design, chromosome position, number of alleles and fragment size ranges have been described previously (Pedersen, Liu, Greenfield, & Echols, 2012). Polymerase chain reaction (PCR) for this panel consisted of an initial activation step of 10 min at 95°C, followed by 30 cycles of 95°C for 60 s, 56°C for 30 s and 72°C for 60 s. A further panel consisting of 14 tetranucleotide STR microsatellite markers and a marker for the SRY gene was also utilized. Primer design and PCR conditions were as previously described (Wictum et al., 2013). Polymerase chain reaction was performed using a 9800 Fast Thermal Cycler (Life Technologies, Johannesburg, South Africa), followed by capillary electrophoresis by an ABI 3500 XL Genetic Analyser (Life Technologies). Fragment sizes for each marker were evaluated using the software program STRand Version 2.4.49 (University of California, Davis, CA, USA; Toonen & Hughes, 2001).

## 3 | RESULTS

Twins A and B were phenotypically normal males. At birth, twins A and B weighed significantly less (*t* test; $p < .001$) than their five littermates; however, this difference had lost statistical significance by the age of 6 weeks ($p = .32$; Table 1). Although similar in physical appearance, they showed slight differences in terms of the size and shape of white markings on the chest, lower legs and the tip of the tail (Fig. 2).

**TABLE 1** Weights of twins A and B and their littermates at birth and at the age of 6 weeks

| Puppy | Weight (g) at birth | Weight (kg) at 6 weeks of age |
|---|---|---|
| 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[a] | 5.7[a] |
| Mean (Non-twins) | 758[b] | 6.1[a] |

Means bearing different superscripts within a column differ significantly ($p < .05$).

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

## 4 | DISCUSSION

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

**FIGURE 2** Monozygotic twins A and B photographed with their dam at 6 weeks of age. Note the differences in the white markings on the chest and paws

**TABLE 2** Genetic profiles derived from seven littermates including monozygotic twins A and B

| Locus | Light female | Brindle male | Brindle female | Dark brindle male | Dark brindle female | Twin A[a] | Twin B[a] |
|---|---|---|---|---|---|---|---|
| AHT121 | 104 | 96,104 | 96,104 | 96,104 | 96,104 | 96,104 | 96,104 |
| AHT137 | 131 | 131 | 131 | – | 131 | 131 | 131 |
| AHTh130 | 129 | 129 | 129 | 129 | 129 | 129 | 129 |
| AHTh171 | 219 | 219 | 219 | 219 | 219 | 219 | 219 |
| AHTh260 | 244 | 244 | 244 | – | 244 | 244 | 244 |
| AHTk211 | 91 | 91 | 91 | 91 | 91 | 91 | 91 |
| AHTk253 | 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 |
| REN105LO3 | 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 |
| REN247M23 | 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 | 17.3,18.3 | 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 |

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

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

placental site during caesarean section, provides strong evidence for monozygosity.

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

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

Conjoined monozygotic twins are believed to arise from the incomplete splitting of an embryo after formation of the primitive streak has begun. In humans, one in 400 monozygotic twins are reportedly conjoined (Hall, 2003). According to Gupta, Lall, and Bajpai (2001), 1%–2% of human conjoined twins are asymmetric (referred to as heteropagus). Logrono, Garcia-Lithgow, Harris, Kent, and Meisner (1997) found that, in a case of human heteropagus conjoined twinning, the parasite and autosite were dizygous, presumably resulting from the fusion of two conceptuses. Thus, conjoined twins may be monozygotic due to fission, but need not be. Conjoined twinning has been reported rarely in the dog (House et al., 2012; Mainland, 1929; Mazzullo et al., 2007; Nottidge et al., 2007; Paquet et al., 2011), and no DNA analyses were performed in the described cases. Nevertheless, the small number of cases of conjoined twins in dogs reported in the literature, most of which describe symmetrical conjoined twinning involving a degree of posterior duplication, suggest that monozygotic twinning in the dog is rare or that splitting events giving rise to conjoined monozygotic twins are rare in this species.

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

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

For genetic identification and parentage analysis purposes, this study shows that dogs with identical genetic profiles, although likely rare, do exist. Bitches may have more conceptuses in the litter than they have corpora lutea (Andersen & Simpson, 1973; Bysted, Dieleman, Hyttel, & Greve, 2001). One cause for this may be multiovular follicles (Reynaud, Viaris de Lesegno, Chebrout, Thoumire, & Chastant-Maillard, 2009; Telfer & Gosden, 1987) from which more than one oocyte may be fertilized. The current case confirms that monozygotic twins is another possible reason for finding more conceptuses than corpora lutea in bitches.

## 5 | CONCLUSION

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

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### CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

### AUTHOR CONTRIBUTIONS

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

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