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Monozygotic origin of bovine and buffalo calves with diprosopia or dicephalia

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ABSTRACT

Congenital malformations, including cephalic anomalies such as diprosopia and dicephalia, represent rare but significant developmental abnormalities in humans and animals. While these conditions are primarily attributed to incomplete embryonic splitting, their genetic origins in domestic animals remain poorly understood. This study presents a detailed molecular and anatomical investigation of three cases of cephalic malformations in ruminants: two Holstein-Friesian calves and one Mediterranean Italian River Buffalo. Anatomical analyses classified the malformations as diprosopus in two cases and dicephalic in one. Notably, this is the first reported case of dicephalia in a female Mediterranean Italian River Buffalo. Molecular analysis using microsatellite markers and sex-linked genes (*AMELX/Y* and *SRY*) confirmed the monozygotic origin and the female sex of all cases, with identical genotypes detected in both heads of each calf.

Congenital malformations can be caused by several mechanisms, including chromosome or gene mutations and environmental factors (eg, toxins) affecting in-utero development [1]. Cephalic parapagia is a rare form of such malformations, and it refers to twins with the union of the trunk and separate heads or some structures of the heads. It is classified as diprosopia when there is a single head with a variety of duplications of craniofacial structures and organs, and dicephalia if there are two totally separate heads [2,3].

It is generally accepted that the incomplete splitting of an early embryo is responsible for the development of conjoined twins, while the fusion of two embryos is considered an unlikely mechanism [4]. There are three main hypotheses considered regarding the formation of *diprosopia/dicephalia*. The first one, known as Spencer (or fusion) hypothesis, states that the embryo is completely divided into two individuals and then rejoined. The second one, known as Carles (or fission) hypothesis, assumes that during early developmental stages the embryo undergoes partial fission. Finally, the Bidondo (duplication) hypothesis suggests that duplication of the early primitive node disrupts right-left polarity, causing divergent signals at the midline and random duplication of tissues with either right or left laterality [5]. In humans, *diprosopia/dicephalia* occurs with a low incidence, 1:50,000–1:100,000 new-born [6]. In livestock the majority of the cases were observed in cattle and buffaloes (Supplemental Table 1), where its incidence is estimated to be 2.2–10% of all congenital anomalies in newborn cattle [7,8]. Similar abnormalities have also been reported in other domestic species, including pigs [9,10], sheep [11] and cats [3,12].

Most reports on conjoined twins have focused primarily on their anatomical characteristics. To the best of our knowledge, no genetic studies confirming the monozygotic origin of *diprosopia/dicephalia* in domestic animals have been reported to date. Therefore, the main aims of the present study are (1) to describe the anatomical characterization, and (2) determine the molecular analysis of microsatellite DNA markers and the X- and Y-linked genes of these two-headed calves.

Three calves with head malformations were studied: two live-born bovine Holstein-Friesian calves (Italian Holstein-Friesian [IHF], case 1; and Polish Holstein-Friesian [PHF], case 2) and one spontaneously aborted Mediterranean Italian River Buffalo (MIRB, case 3). No ethical approval was required in compliance with European Directive 2010/63/

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EU and Italian Regulation (D.Lgs n. 26/2014), because all the data derived from animals was submitted under the diagnostic scheme of the Department.

Case 1. A newborn female IHF bovine calf (Fig. 1a) that was transported immediately after calving to the Veterinary Teaching Hospital of the Department of Veterinary Medicine and Animal Production of the University of Naples Federico II (Italy) due to the abnormal head conformation. The calf showed generalized muscular hypotrophy and was unable to lift or move its head; a detailed clinical presentation is reported in Supplementary file 1. Briefly, neurological examination showed tetraparesis associated with uncoordination ataxia and weak non-voluntary movements. Proprioception and postural reactions were completely absent. Spinal reflexes were reduced on all four limbs, and palpation of the vertebral column elicited no pain. Examination of the head showed a lack of reaction for all the cranial nerves. During the examination it was was impossible to evaluate the tone of the tongue or sucking reflex due to the reduced range of motion of both temporomandibular joints. Moreover, the swallowing reflex was absent when evoked by external oropharyngeal area stimulation. Physiological nystagmus, as well as the vestibular eye drop response after dorsoextension of the heads, were bilaterally absent because of permanent dorsolateral strabismus involving all the eyes.

Computed tomography (CT) revealed a complex morphology for the two partially fused heads. Each head had its own eyes, nasal cavities and mouth (Fig. 2a), which partially fused medially at the temporomandibular joint (Fig. 2b). The bones of the neurocranium fused at the level of the parietal bones, with a single enlarged basisphenoid and occipital bones, and a "Y"-shaped presphenoid bone (Figs. 2c and d). Each head had a complete, normal forebrain with two lateral ventricles, a single third ventricle and mesencephalic aqueduct (Fig. 3a). Both heads had a common hindbrain with a single, centrally located cerebellum as well as a single pons and medulla oblongata, this last passing through an abnormally enlarged foramen magnum (Fig. 3b). Moreover, the atlas appeared enlarged (Fig. 2d), while the remaining cervical vertebrae were single and showed normal morphology. No other abnormalities were found elsewhere.

On post-mortem examination, the calf showed complete duplication of the head with partial fusion of the cranium at the level of parietal bones, complete muzzle duplication, tetrophthalmos (Fig. 4a) and two normally developed ears (Figs. 4a and b). In the cranial cavity, two completely duplicated and morphologically normal brains were observed (Fig. 4a). Both brains were fused at the caudal portion of the mesencephalon (Figs. 4c and d) with two fused and moderately oedematous cerebella. Two olfactory and optic nerves and a single morphologically normal spinal cord were also observed. Moreover, the oral and nasal cavities were duplicated with two partially separated tongues converging into a single laryngopharynx. Trachea and lungs were morphologically normal (Fig. 4d). No additional macroscopic malformations were observed in the other assessed organs, confirming the CT findings. Brain samples were collected for routine histological examination, ~~examination were~~ fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 4 μm and stained with haematoxylin and eosin (HE). Mild to moderate histopathological alterations were predominantly confined to the cerebellum. These changes consisted of multifocal thinning of the external granular layer and segmental Purkinje cell loss, with the formation of characteristic 'empty baskets' within the cerebellar cortex. Occasionally, neurons of the grey matter of both the cerebral and cerebellar cortices showed shrunken eosinophilic cytoplasm and pyknotic nuclei. Multifocal meningeal haemorrhages were also present in the leptomeningeal spaces. Diffusely, in the brain parenchyma there were empty spaces surrounding blood vessels, sometimes filled by amorphous and slightly eosinophilic fluid (Fig. 5).

Case 2. A female PHF bovine calf with abnormal head conformation that was delivered on a small farm in the western part of Poland. The calf was unable to stand and was euthanized on welfare grounds shortly after birth. The head showed a single neurocranium, four eyes, four nostrils, one pendolous ear on each side (laterally) and two mouths (Fig. 1b). No further post-mortem investigations were carried out.

Case 3. A late-term abortion female MIRB calf that was referred to the Department of Veterinary Medicine and Animal Production of the University of Naples Federico II (Italy) due to the abnormal head conformation [13]. In particular, the calf showed two phenotypically complete heads with two brain cases, four eyes, four nostrils, two dropping ears per head and two muzzles. CT examination confirmed the presence of two fully developed and separated skulls (Figs. 6a and b). Each head had normal facial and neurocranium structures, as well as a normal atlanto-occipital joint. The two spinal cords and columns merged at the level of C2, with a "Y"-shape (Figs. 6c and d). Multiple gas bubbles (interpreted as post-mortem changes) were observed within and around the brain parenchyma, spinal cord and soft tissues of the head and neck. On post-mortem examination, the cadaver showed complete duplication of the head with complete muzzle duplication, tetrophthalmos and four normally developed ears. In the cranial cavity, two completely duplicated and moderately oedematous brains were observed. The meninges were moderately hyperaemic. Two completely separated tongues and larynxes were also detected. No additional macroscopic malformations were observed in the other organs examined.

Tissue samples for DNA isolation were collected *post mortem* from all cases (brain samples from both heads in cases 1 and 3; fragments of ears and hair follicles in case 2). Comparative molecular studies of samples collected from both heads were performed. Searching for the presence of

Figure 1. Heads of two diprosopus bovine calves. (**a**) case 1. (**b**) case 2.

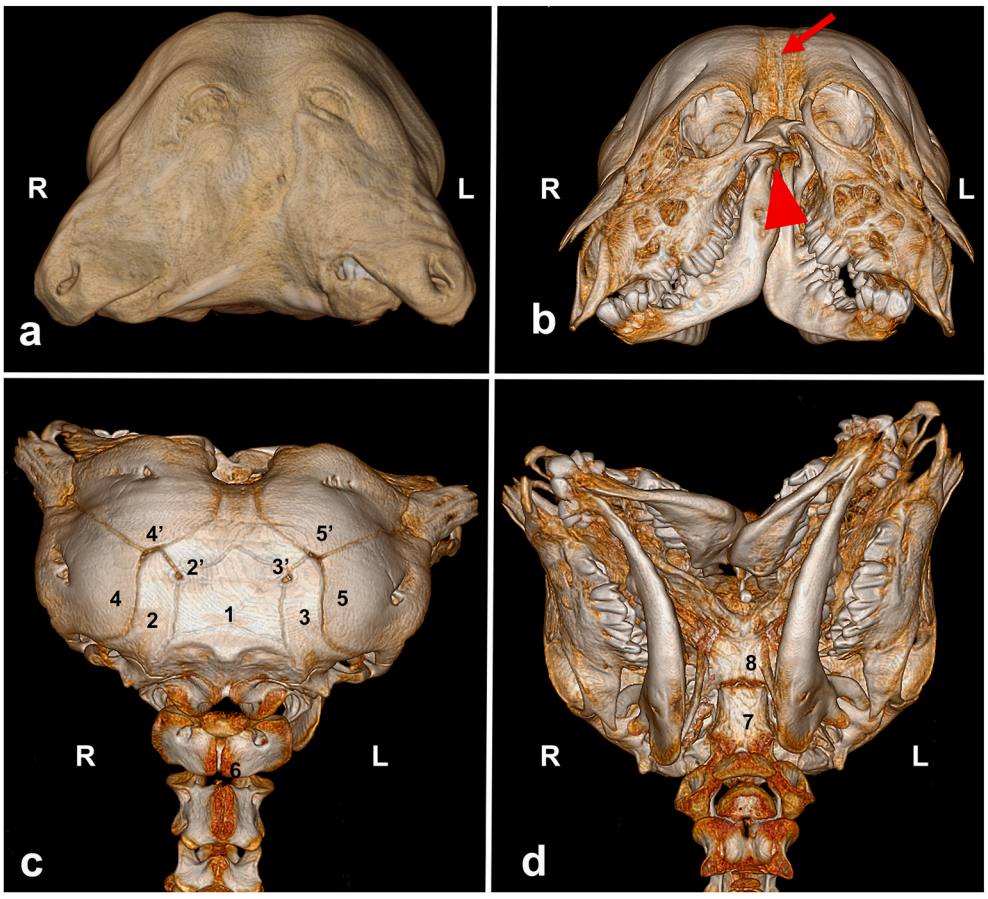

Figure 2. Three-dimensional volume rendering of case 1. Frontal (**a**, **b**), dorsal (**c**) and ventral (**d**) views. **(a)** Each head has normal eyes, nasal cavities and mouth, which fuse together on their caudomedial aspect. **(b)** There is a partial fusion of the temporomandibular joint (red arrowhead) and parietal bones (red arrow). **(c)** Each head has normal paired frontal bones (4–4' and 5–5'), partially developed parietal bones (2–2' and 3–3'), fusing medially, and an irregularly shaped single occipital bone (1). **(d)** From the ventral view, the single enlarged basisphenoid (7) and the "Y"-shaped presphenoid (8) bones are clearly visible.

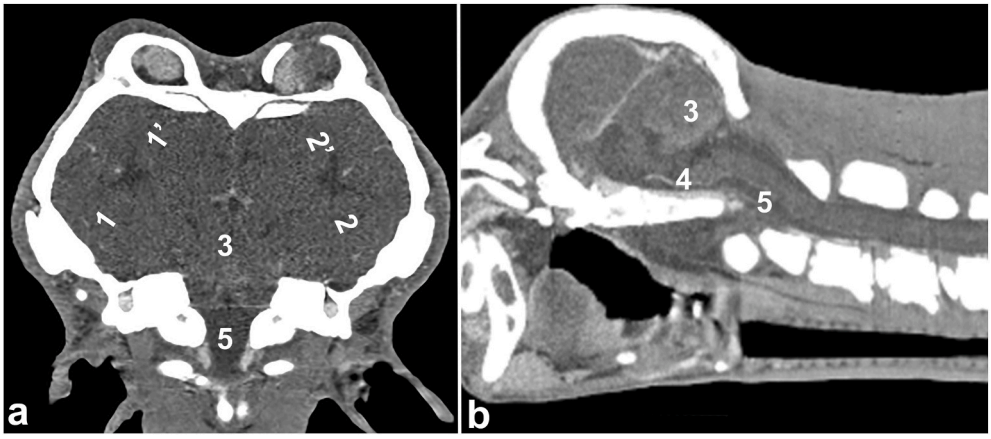

Figure 3. Multiplanar dorsal/transverse oblique CT reconstruction of case 1 of the neurocranium at the level of the parietal lobes (**a**) and multiplanar sagittal oblique CT reconstruction of the craniocervical junction (**b**). Each head has a complete, normal forebrain with normal frontal and parietal lobes (1–1' and 2–2'), while there is a common hindbrain with a single cerebellum (3), pons (4) and medulla (5).

Y-linked genes (*SRY* and *AMELY*) and X-linked genes (*AMELX*) was performed using the polymerase chain reaction (PCR) technique. The primer sequences and lengths of the amplicons are shown in the Supplementary material This approach was applied to detect molecular sex (male – presence of the Y-linked genes, female – lack of the Y-linked genes and presence of the X-linked gene). A panel of 12 bovine microsatellite (STRs – short tandem repeats) and 14 buffalo microsatellites (STRs) was used to determine whether both heads have the same genotype (monozygotic origin) or different genotypes (dizygotic origin). The lists of the studied STRs are shown in Table 1.

Detailed anatomical investigation of cases 1 and 3 allowed us to classify them as diprosopus and dicephalic, respectively. Case 2, in which anatomy was not investigated in detail, was tentatively classified as diprosopus. Therefore our study diagnosed two new cases of

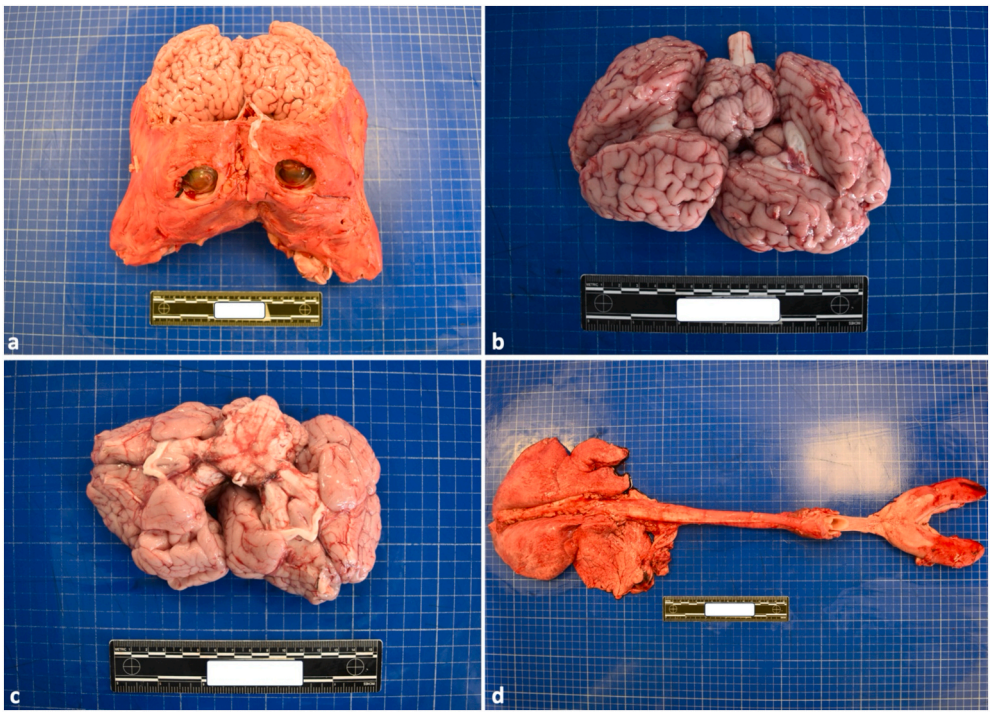

Figure 4. Anatomopathological findings of case 1. (**a**) Head duplicated and fused at the parietal bones, with a single enlarged occipital bone. (**b, c**) Brains fused at the caudal portion of the mesencephalon. (**d**) Two partially separated tongues and a single and morphologically normal larynx, trachea, and lungs. (All photographs of the specimens were taken against an underlying metric grid, with each square measuring 1 cm per side.)

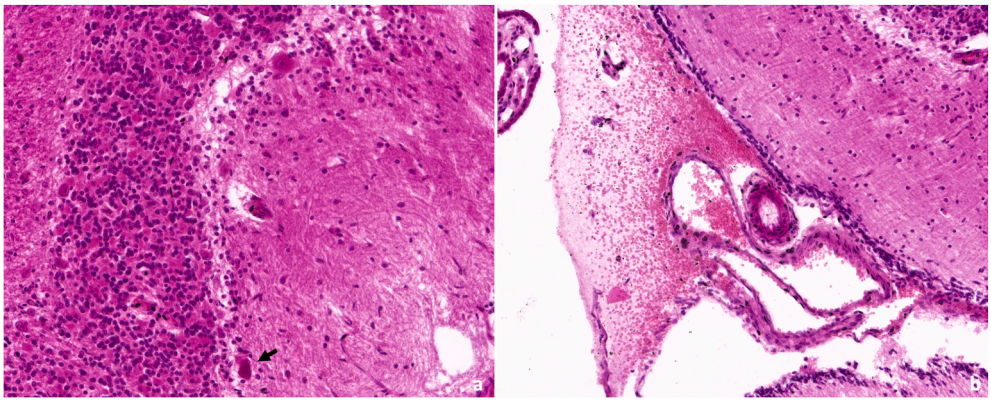

Figure 5. Histological features of cerebellum of case 3. (**a**) Segmental depletion of Purkinje cells associated with persistent Purkinje cells showing shrunken eosinophilic cytoplasm and pyknotic nuclei (arrow). (**b**) Acute haemorrhages. HE. ×20.

diprosopus calves in Holstein-Friesian cattle and the first case of dicephalia in the MIRB. A predominant occurrence of diprosopia in Holstein-Friesian breed (Supplementary Table 1) and reports of its familial occurrence in German Holstein-Friesian cattle [10,14] may indicate a heritable predisposition to the onset of this condition in this breed. It is noteworthy that our study confirmed a more frequent occurrence of this abnormal condition in females (Supplementary Table 1). This may indicate an early embryonic death of the affected male co-twins [7].

Histological data of different tissues collected from *disopropia/dicephalia* calves are very scarce. Biasibetti *et al* [7] examined four calves affected by diprosopia; while the cerebral hemispheres and brainstem appeared histologically normal, two individuals had specific alterations in the cerebellar cortex characterized by thin layers of white matter enveloped by moderately disorganized grey matter. These authors also studied one dicephalic calf, but no histological abnormalities were found. McNulty *et al* [5], in a mixed-breed beef calf with symmetric parapagus diprosopus tetrophthalmos, reported that the histological patterns of samples of lung, liver, kidneys, spleen, heart, skin, cerebellum, brainstem and globes were within normal limits. Our study of case 1 showed lesions similar to those observed by Biasibetti *et al* [7] in two cases; however, additional alterations, most notably the depletion of Purkinje cells, were also found. This finding would explain the observed clinical signs, such as tetraparesis and motor alterations.

Studies on the aetiology of human conjoined twins have suggested that this condition arises at an early stage of embryonic development, approximately 13 days after fertilization, and has a monozygotic origin [15]. A very rare form is heteropagaus (parasite) twins, in which a severely defective (parasite) co-twin of monozygotic origin is observed [15]. Interestingly, there is only a single report of a dizygotic case, confirmed by molecular analysis [16].

Our molecular study supported the monozygotic origin of conjoined neonates or fetuses. In all cases, only the X-linked gene (*AMELX*) was observed, while the Y-linked genes (*SRY* and *AMELY*) were absent (Figs. 7 and 8). To analyze the genetic origin of both heads, we used a set

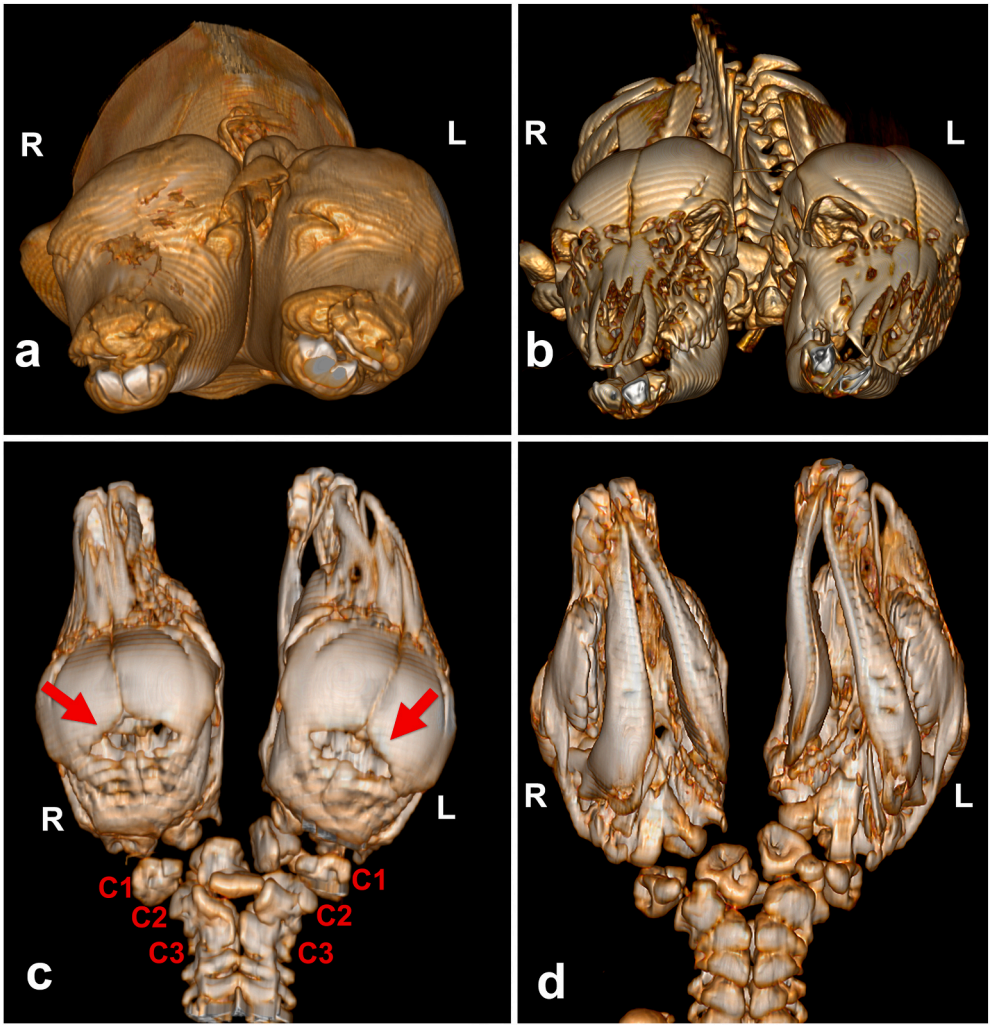

Figure 6. Three-dimensional volume rendering of case 3, showing frontal (**a**, **b**), dorsal (**c**) and ventral (**d**) views. (**a**, **b**) The two heads are completely developed and separated. (**c**) The occipital fontanelle are open as normally expected (red arrows). (**c**, **d**) The vertebral column has a "Y"-shape separating into two segments cranial to C2, with each head having a normal atlanto-occipital joint.

Table 1
Genotypes at the analyzed STR loci.

Cattle			Buffalo	
STR loci	Genotypes		STR loci	Genotypes
	Case 1 (IHF)	Case 2 (PHF)		
BM1818	262/266	262/266	CSSM60	120/125
BM1824	178/180	178/188	BMC1013	225/231
BM2113	135/139	139/139	CSSM47	132/147
ETH3	129/129	129/129	INRA026	92/96
ETH10	223/225	209/213	CSSM19	136/136
ETH225	148/148	148/148	BMO922	69/75
INRA23	214/214	202/202	RM4	156/157
SPS115	248/248	248/252	INRA006	119/121
TGLA227	89/91	89/89	CSSM42	182/184
TGLA122	143/143	142/163	MAF65	116/119
TGLA126	115/117	117/117	D5S2	215/215
TGLA53	158/184	160/184	CSSM38	182/184
			BM1706	229/246
			CYP21	184/186

of microsatellite markers recommended for parentage control by the International Society for Animal Genetics (ISAG). It was found that samples isolated from both heads of each case had the same genotype at all STR sites (Table 1).

To the best of our knowledge, molecular studies of conjoined twins in domestic animals have not been performed to date However, there is a report concerning four amorphous globosus fetuses, which were observed along with viable fetuses delivered by three cows. Molecular analysis, with the use of 17 STR markers, showed that the affected fetuses had the same genotype as phenotypically normal co-twins; thus, the normal and amorphic fetuses had monozygotic origins [17].

In conclusion, this study supports the monozygotic origin of cojoined bovine and buffalo twins based on molecular marker analysis. It remains intriguing that the cases described in this study, as well as almost all earlier reported bovine and buffalo calves, were female.

Statement of author contributions

MS and **SA** conceived the study; **SA**, **MS**, **LM**, **PC** and **FC** designed the experiments; **JNW**, **GB**, **ED** and **GP** collected the samples; **JNW**, **MGR**, **ED** and **GP** performed the experiments; **JG** and **DC** collected clinical data; **MS**, **SA**, **FC**, **VP**, **JG**, **GP** and **LM** helped with the interpretation of the pathological and experimental findings and provided valuable insights based on their experience with natural or experimental cases of this disease; **MS**, **SA**, **PC** and **LM** wrote the first draft of the manuscript. All authors reviewed, edited and approved the final version.

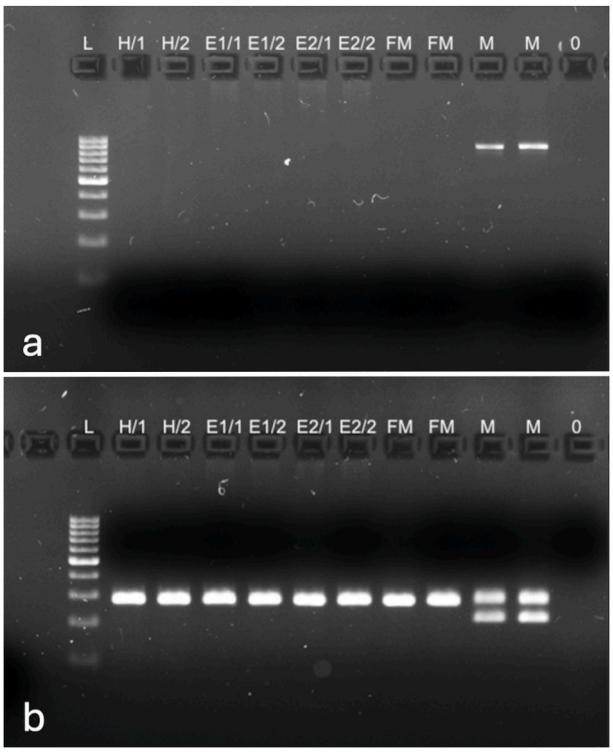

Figure 7. Agarose gel electrophoresis of PCR amplicons representing X- and Y-linked bovine genes in case 2 (PHF) and control females (FM) and males (M). (**a**) amplification of *SRY* gene. (**b**) amplification of *AMELY* and *AMELX* genes. L – 100 bp DNA ladder, H – DNA from hair follicles, E1 and E2 – DNA from both ears, 0 – negative control (sample with no DNA template). For each sample PCR was performed in duplicate.

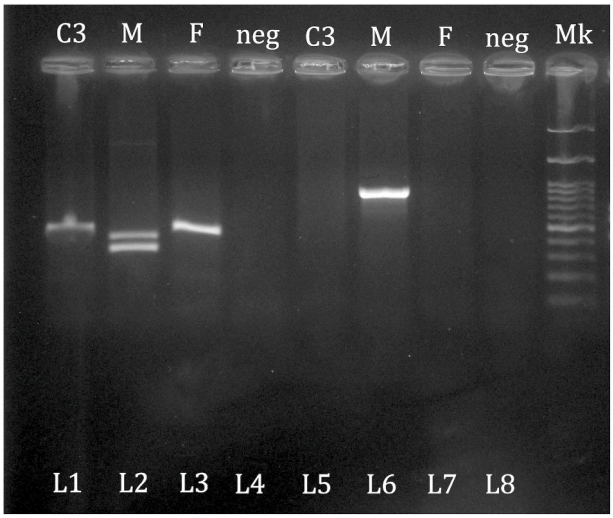

Figure 8. Agarose gel electrophoresis of PCR amplicons of *AMELX/Y* gene (Lane 1- 4)and *SRY* gene (Lane 7–8) in MIRB: C3, case3; M, control male; FM, control female; neg, negative control (sample with no DNA template); MK, 100 bp DNA ladder.

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Declaration of competing interest

The author(s) declared no conflicts of interest with respect to the research, authorship and/or publication of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcpa.2026.02.004.

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