FIRST MENTION OF A WOLF-PACK WITH BLACK PHENOTYPES IN PROVENCE (SAINTE-BAUME, FRANCE): GENETIC INVESTIGATION OF A SUSPECTED HYBRIDIZATION EVENT

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First Mention of a Wolf-Pack with Black Phenotypes in Provence (Sainte-Baume, France): Genetic Investigation of a Suspected Hybridization Event. Roda, F., Philiber, J.-N. — We document the first appearance of a wolf pack with black individuals in France. Black-coated wolves are excessively rare in Europe and are usually suspected of being wolf × dog hybrids. Camera trapping (CT) was used to characterize the phenotypes of the wolf pack's grey and black wolves. We used a detection dog to rapidly identify and noninvasively collect fresh wolf scats. The male wolf of the breeding pair exhibited the typical phenotype of a grey wolf, whereas the reproducing female was black-coated. Four of the six pups were black, and two were grey. Analysis of microsatellite markers, using French wolves genotypes as reference, revealed that none of the wolves were first or second generation hybrids. In accordance with previous studies, we conclude that atypical phenotypes make it possible to suspect wolf × dog hybridisation, but genetic analyses remain necessary to assign an individual canid to a genetic class.

Key words: *Canis lupus*, (wolf-dog) hybrid, hybridisation, large carnivore, detection dog, faecal samples, camera trapping, genotype.

Practitioner Points

Black-coated wolves are excessively rare in Europe and are usually suspected of being wolf × dog hybrids.

   We used a detection dog to rapidly identify and noninvasively collect fresh wolf scats.

    Analysis of microsatellite markers, using French wolves genotypes as reference, revealed that none of the wolves were first or second generation hybrids.

Introduction

Wolves are recolonising parts of their historic range in the French Alps, following the first official confirmation of the species return in the early 1990s in this area (Peillon & Carbone 1993; Valière et al. 2003). In France, wolf recovery is occurring in areas of intensive sheep breeding activity. In these regions, livestock depredation attributed to wolves can be considerable (> 12,000 heads in 2019), causing substantial economic and social impact (Données DDT(M)-DREAL Auvergne Rhône-Alpes 2019). The conflict over wolves is particularly prominent in areas of recent wolf recolonisation as people are unaccustomed to sheep depredation and herds are not often well protected (Chapron et al., 2014).

    Recently, wolf hybridisation has become a very hot topic in France (Reportage France 3, 2017). Wolf × dog hybridisation occurs regularly in Europe (Vilà et al., 2003; Godinho et al., 2011; Randi et al., 2014; Kusak et al., 2018) and 3.6% of French wolves are F1 hybrids and an additional 7.2% are carriers of dog genes but potentially with distant ancestry (Duchamp & Queney, 2018). European authorities recommend eliminating wolf × dog hybrids, as introgression of dog genome in wolf population may constitute a serious conservation concern (Council of Europe, 2014). Based on this European recommendation, a collective of sheep farmers and politicians contested the results of the wolf population monitoring carried out by the French authorities. Wolf opponents claimed that all French wolves are hybrids (ON-CFS, 2018); politicians proposed accordingly to eliminate them (Sénat, 2017).

    In this political context, wolves with atypical phenotypes are usually suspected of being hybrids. In general and with consensus in Europe, an atypical phenotype may suggest the occurrence of wolf × dog hybridisation, but genetic analyses remain necessary to reliably assign an individual canid to a genetic class (Wolf, dog or hybrid; Galaverni et al., 2017; Pilot et al., 2018; Donfrancesco et al., 2019). In North America, black wolf phenotypes are common and may locally represent 50% or more of the population (Gibson et al., 2002; Hedrick et al., 2014). In contrast to the situation in North America, the black phenotype is rare in Europe: based on sampling of 700 wolves made in Russia, Belarus, Ukraine, Lithuania and Bulgaria, only one wolf was found with a black coat (W. Jedrzejewski, as reported in Apollonio at al., 2004); in a recent study Kusak et al. (2018) showed that a few samples collected in Croatia corresponded to black phenotypes. An exception is represented by the Italian northern Apennines, where black wolves occur locally at a non-negligible frequency (Apollonio et al., 2004; Caniglia et al., 2013). The genetic base of the black coat in wolves is a 3 nucleotides deletion at the β-defensin gene *CDB103* that could be of domestic dog origin and therefore a signal of hybridisation (Candille et al., 2007). According to Galaverni et al. (2017), the black coat was also found in canids with
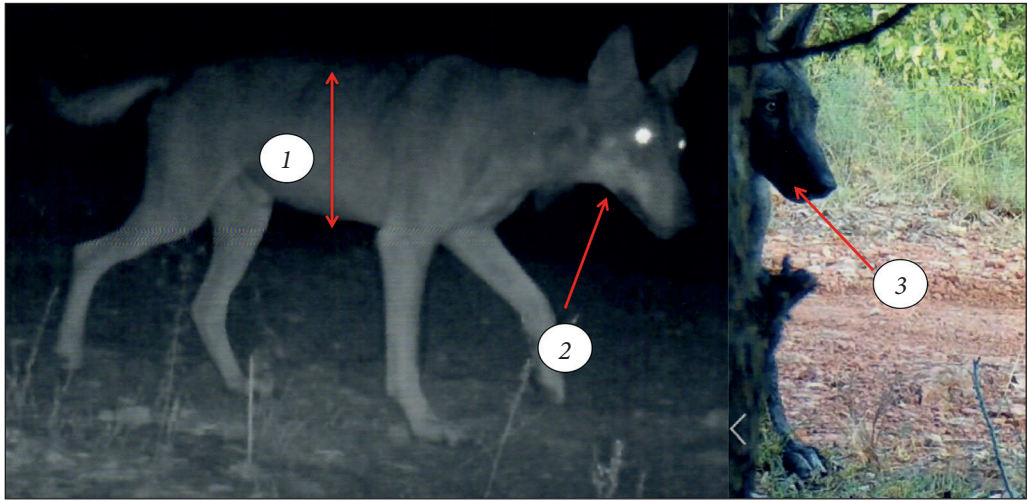
Fig. 1. First photographs of the breeding adults of the Sirius Black pack. On the left the male has a typical grey wolf colouration contrast between the dark back (*1*) and lighter belly and white face mask (*2*). The female has a black coat and does not have a white face mask (*3*). The phenotype alone of the female does not make possible to determine whether it is a wolf with atypical coat, a shepherd type dog or a wolf × dog hybrid. © Jean-Noël Philibert

genomic profiles that clustered with wolves and showed no sign of dog ancestry, so genetic analyses are therefore needed to determine whether a black individual shows any recent dog ancestry. From 1992 to 2016, French authorities (ONCFS, 2016) detected no wolf with "atypical phenotype" (sensu Kusak et al., 2018). Between 2016 and 2019, sheepherders and game hunters reported sporadic sights of wolves with atypical phenotypes, but there were no photographs to support these observations. In 2019, a black female wolf was killed at "La Malle" (Alpes Maritimes) and the expertise report concluded that the canid was a wolf without any sign of dog admixture (ONCF, 2019). This female wolf exhibited a w22 haplotype (typical from Italian wolves), and it is likely that this female may have originated from Italian northern Apennines.

During the summer of 2021, previously unrecorded male and female wolves were photographed in the Sainte-Baume Regional Park. The male exhibited a typical grey wolf phenotype, whereas the female was black-coated (Fig. 1). Because the visual analysis of the available photographs of the female phenotype did not allow a reliable discrimination between a wolf, a shepherd type dog or a dog x wolf hybrid, the Fédération Départementale des Chasseurs du Var placed a set of Camera traps (CT) on the area, to monitor the wolf couple presence and identify possible reproduction events. In a previous study (Roda et al., 2022), CT captured no free ranging dogs during 9 months of monitoring (or during this study), so we assumed that there were no free ranging dogs in the Park. Thanks to the surveys, at the end of August, in the territory of the Sainte-Baume regional Park, six young pups were detected, four of them showing the atypical black coat (Fig. 2). Within the same litter, animals with the typical grey wolf pattern coexisted with others with a melanic coat (Fig. 3). This was the first time, in France, that a pack consisting of several wolves with atypical phenotypes has been identified and investigated.
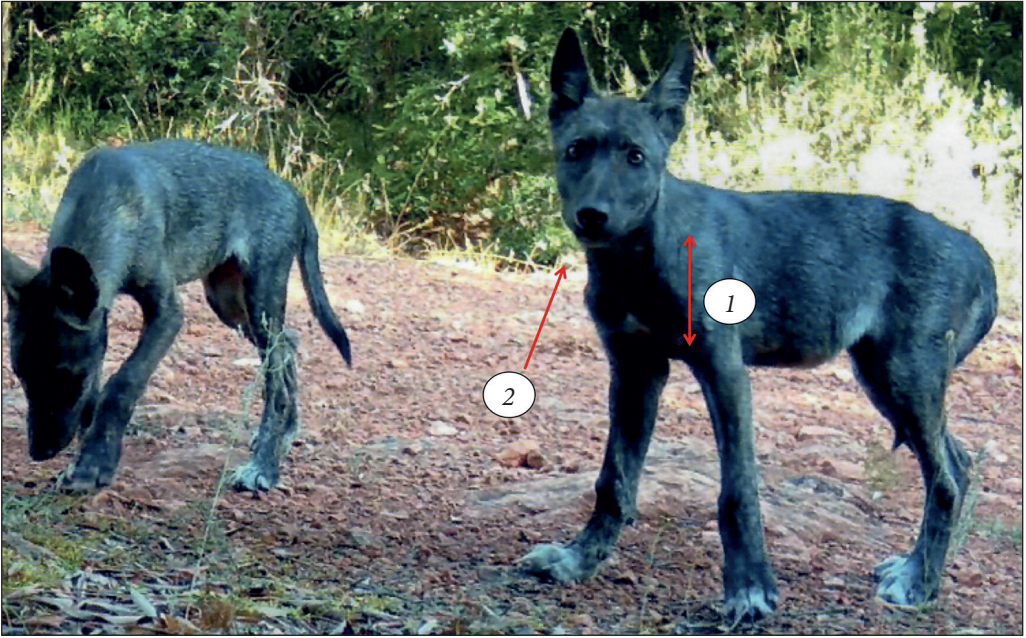
Fig. 2. Two pups with atypical black coats (Sirius Black pack). The black colour of the coat (*1*) is uniform in these pups, there is no contrast between the back and the belly and the white face mask (*2*) is absent in these pups, as in their parent female wolf. Coat may be described as "bluish grey" (right pup) or "silver-black" (left pup). © Jean-Noël Philibert
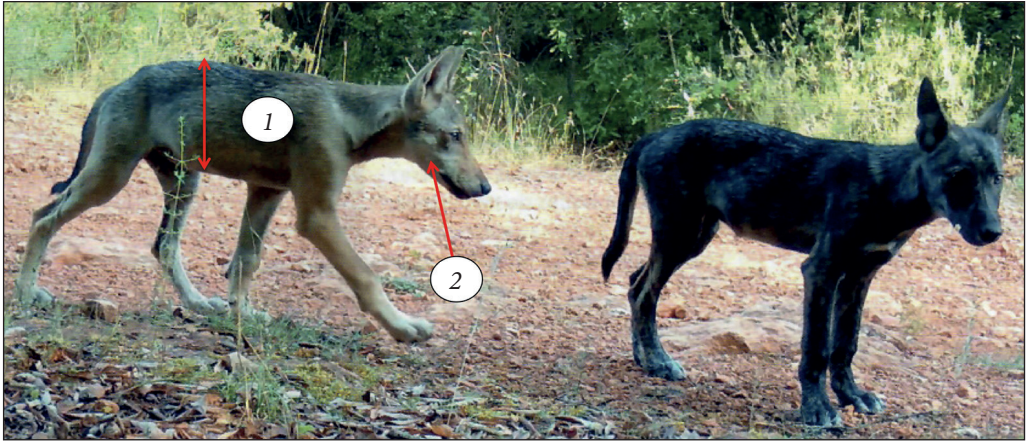

Fig. 3. Two pups of the same litter with different phenotypes (Sirius Black pack; photograph taken in August). The left pup has a typical grey wolf phenotype with marked contrast between back and belly (*1*) and a white face mask (*2*). The second pup (right) has an atypical uniform black coat. © Jean-Noël Philibert

We used a dog trained to detect wolf scats to rapidly and non-invasively collect fresh fecal samples for genetic analysis, and conduct a more intensive and accurate investigation of the newly established pack, called "Sirius Black". To avoid polemics and allow comparisons with genotypes published by French authorities, we choose to send our samples to ANTAGENE, the same private laboratory used by the French Office of Biodiversity (ONCFS, 2018; Duchamp & Queney, 2018).

Material and Methods

We used a trained detection dog to find fresh scats, following a protocol previously described in Roda et al., 2021 and Roda et al., 2022. Briefly, the team surveyed transects that were preferably circular for logistical reasons (return to the vehicle). The Sainte-Baume Regional Park (43°32 N; 5°83 E; https://inpn.mnhn.fr/espace/protege/ FR8000053) is home of three referenced wolf packs (Lupi, Neowise, Vega), in addition to the recently established wolf pack (Sirius Black). Wild boar abundance varies significantly throughout the massif (Roda & Roda, 2024), with high numbers harvested (6.3 wild boars killed/km²/year). In contrast, roe deer (*Capreolus capreolus*) are scarcer (0.9 animals killed/km²/year. A small cluster of approximately 50–100 fallow deer (*Dama dama*) and red deer (*Cervus elaphus*), has been recently introduced by game managers (Krammer, 2016; Roda, 2016). Livestock animals are absent in the prospected area. The first wolf reproduction occurred in 2020 (Poulard et al., 2021). The marking sites of the wolf packs are well-known (Fig. 4), have been extensively studied (Roda et al., 2022) and individual wolves were recently genetically identified (unpublished data). Twenty scat samples were collected at two dates (26th and 30th October 2021) stored individually in a frozen sealed plastic bags and analyzed for 22 markers. Genetic analyses were performed by a subcon-
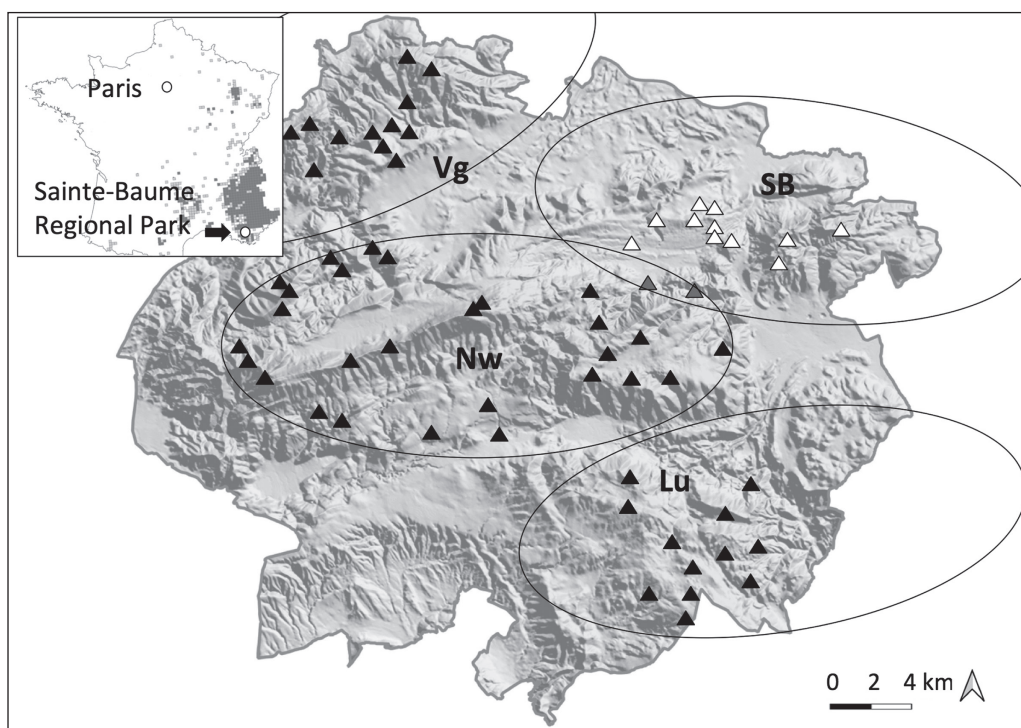
Fig. 4. Study area, showing the territory of the four wolf packs present in the Sainte-Baume Regional Park. The administrative boundaries of the Park are shown in light grey. White triangles indicate marking sites where scats of the "Sirius Black" wolf pack were collected, black triangles indicate marking sites of three adjacent wolf packs. Red triangles indicate marking sites at the edge of two adjacent wolf packs (Sirius Black and Neowise) where scats were collected. Nw: Neowise wolf pack; Lu: Lupi wolf pack; SB: Sirius Black wolf pack; Vg: Véga wolf pack. Marking sites of the Lu, SB and Vg packs outside Park boundaries are not shown

tracted private laboratory (ANTAGENE, https://www.antagene.com/en). We chose to use this laboratory because it is the one that has been used for years by the French Office of Biodiversity in France, so that the genotypes identified in our study can feed the database and population model of the French Office of Biodiversity. All samples were analyzed using a mitochondrial DNA (mtDNA) species identification test, as well as a polymerase chain reaction (PCR) multitube approach based on 22 micro-satellite markers: AHT103, AHT111, AHTk211, CPH02, C09.173, CPH05, CFX30371, CXX.279, FH2161, FH2054, Dbar1, REN162C04, FH2010 & C27.442 (Godinho et al., 2015); FH2096, FH2088, FH2004, FH2140 & FH2137 (Francisco et al., 1996), C09.250 (Ostrander et al., 1993), PEZ17 (Neff et al., 1999), INU030 (Ji et al., 2007) and one sex-specific marker for individual gender identification (AMEL, Ji et al., 2007). Extraction methods, PCR protocols and individual identification procedure as well as genotype reliability testing are described in detail in Valière et al., 2003. A quality index (QI) was calculated for each sample by comparing each replicate genotype on each marker to the consensus genotype (Miquel et al., 2006). The QI were then averaged over all repeats for each locus, and then over all loci for an individual to obtain a QI per sample.

To assign individual genetic profiles to their taxon (wolf, dog), a Bayesian clustering approach was implemented. The computer program STRUCTURE v.3.4 (Pritchard et al., 2000; Falush et al., 2003) was used to cluster individuals according to their species with the admixture model, correlated alleles frequencies and prior on reference populations (POPINFO = 1), with a burn-in of 100,000 and 500,000 iterations of the MCMC chain. Twenty repetitions were performed with the number of cluster K set on 2 and probabilities and credibility intervals were averaged over the 20 repeats. As references, ANTAGENE included in the analysis domestic dogs (breeds and village dogs) and grey wolves from the French population. Hybrids were detected based on the assignment probabilities obtained with STRUCTURE (Bohling & Waits, 2011). Individuals whom 95% credibility interval encompassed either 0 or 1 were considered as individuals with no-admixture and the remaining individuals were considered as hybrids. All the genetic procedures and analyses are detailed in Supplementary Material S1.

The genotypes obtained in this study were compared with those from the Database of the French Office of Biodiversity to examine the genetic proximity of Sirius Black wolves with other French wolves. For this analysis (different from the analysis with STRUCTURE provided by the ANTAGENE laboratory, as the data used by ANTAGENE are not freely available) we used the dataset freely provided in Duchamp & Queney (2018), ONCFS (2019) and OFB (2021) for comparison purpose. The sample size consisted of 41 dogs genotypes, seventeen F1 wolf-dog hybrids genotypes, 62 back-cross genotypes and 579 wolf genotypes (OFB, 2021).

Camera trapping was simultaneously conducted in the "Neowise" and "Sirius black" wolf pack territory from 1 June 2021 to 1 January 2022, using 5 cameras. CT use allowed characterising wolf phenotypes. Remote motion-activated cameras were placed on forest roads used by wolves. Each camera trap was active 24 h day-1, 7 days week-1, and was visited by observers at variable intervals (from 2 to 20 days) in order to change batteries and SD cards. All cameras were provided with a passive infrared sensor and LED flash.

The supplementary material (S1: Methods and S2: Genotypes) can be downloaded on the Figshare repository: https://figshare.com/ndownloader/files/51849224.

Results

Wolf phenotypes

CT allowed to regularly capturing images of wolves of the two adjacent wolf packs (Sirius Black and Neowise). Good quality photographs allowed characterising the phenotypes aging-changes of black individuals. As they aged, the young's black coat thinned lightly, displaying a palette of colours between dark silver and grey-blue black. An important contrast appeared between the colour inside the legs (beige as in the typical grey wolves) and the rest of the coat much darker. The belly was also lighter than the back, tending towards beige or red (Fig. 5).

Genetic results

A total of 20 putative wolf scats samples were detected and collected, 17 in the Sirius Black pack area and three at the edge of the territory with Neowise pack. Eighteen (90%) genotypes out of the 20 analyzed scats were assigned to the grey wolf species (mean posterior probability of 0.998; standard error: 0.001); the two last samples were of very poor quality and no species could be identified. Fourteen samples (70%) resulted in reliable multilocus genotypes (see Supplementary Material S2). Seven different wolves were identified. The three samples found at the edge of the Sirius Black/Neowise territory were recaptures of two previously known wolves, the dominant female and male from the adjacent Neowise pack. All other genotypes (11 out of 14) were identified as belonging to five previously unknown wolves from the new
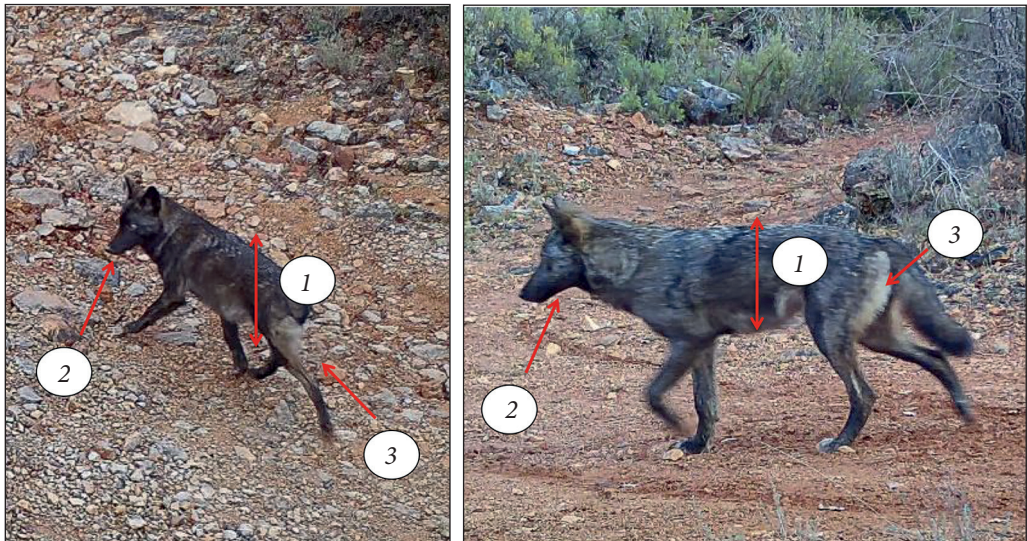
Fig. 5. Two photographs showing young wolves that have reached their adult size (Sirius Black pack). Despite the overall appearance of atypical black coat, there is (*1*) a difference in colour between the back (darker) and the belly (lighter, tending toward beige/red). Adult individuals have (*2*) no white facial mask; the inside of their legs is (*3*) beige as individuals with the characteristic grey wolf phenotype (compare with Figures 3 and 4). © Frédéric Gervais

Sirius Black pack. All haplotypes were already known. Association between camera-trap data and scat genotyping allowed identifying the dominant individuals and the pups. In particular, four samples were identified as belonging to the dominant male and two samples to the dominant female of the Sirius Black. The last 5 samples (out of 14) were identified as belonging to three pups of Sirius Black pack (Table 1). Analyses did not reveal any recent hybridisation (F1 or F2). No samples were associated with a domestic dog breed (*Canis lupus familiaris*). The genotype of the Sirius Black pack's black-coated reproducing female was very close related to that of another black female wolf legally killed in the French Alps (ONCFS, 2019; see Supplementary Material S2).

Discussion

The appearance of black wolves in the Provence region highlights the issue of wolf × dog hybridisation in France. The "black coat" phenotype association with hybridisation has been confirmed in a recent genomic study by Caniglia et al. (2013) conducted in neighbouring Italy, in accordance with previous hypotheses derived from North American wolves suggesting the ancient introduction of dog genome around the genomic region of the β-defensin gene (Anderson et al., 2009). This phenotype is likely a signal of dog-derived genetic introgression in the wolf gene pool. Other studies found black-coated individuals to be genetically wolves (Galaverni et al., 2017). One must keep in mind that our results don't show that black wolves are not hybrids, but that they are not first or second-generation hybrids. The interpretation of data results is greatly sensitive to the sample size of the dog and wolf populations used as reference (see the discussion in Harmoinen et al., 2021; Vik Stronen et al., 2022). In this study, we used the database of French wolves, free-ranging dogs and hybrids provided by the French Office of Biodiversity (OFB, 2021). The methodology used in our study doesn't allow reliable discrimination of backcrosses hybrids from non-ad-

Table 1. **Summary of genetic results**

Identifi-cation	Wolfpack	Sexe	Status	Nb. samples	QI	Assign-ment	Haplo-type	P wolf	<Cl95	>Cl95
Neo 01	Neowise	XY	Dominant	1	0.82	Wolf	w22	0.999	0.985	1.000
Neo 02	Neowise	XX	Dominant	2	0.98	Wolf	w22	0.998	0.978	1.000
SB01	Sirius Black	XY	Dominant	4	0.93	Wolf	w22	0.999	0.986	1.000
SB02	Sirius Black	XX	Dominant	2	0.96	Wolf	w22	0.998	0.972	1.000
SB03	Sirius Black	XY	Pup	1	0.66	Wolf	w22	0.999	0.983	1.000
SB04	Sirius Black	XX	Pup	2	0.99	Wolf	w22	0.995	0.930	1.000
SB05	Sirius Black	XY	Pup	2	0.95	Wolf	w22	0.999	0.985	1.000

Note. Identification: name of each individual wolf. nb. samples: number of genetic scat samples found for each individual wolf. QI: quality index of the sample. If more than one sample available for each individual wolf, the best QI value is shown. Hybridization: hybridization diagnostic (see methods); no hybrids found in this study. Haplotype: haplotype diagnostic. All wolves were from italo-alpine lineage (haplotype w22, *sensu* Pilot et al., 2010). *P* wolf: assignment probabilities obtained with STRUCTURE (Bohling & Waits, 2011). < CI95: inferior 95% credibility interval. > CI95: superior credibility interval.

mixed wolves (VonHoldt et al., 2013); this assertion makes consensus in Europe (Duchamp & Queney, 2018; Pilot et al., 2018). In other words, the dog origin of this (black) phenotype is sufficient to suspect a hybridisation event but a genetic assessment is necessary to assign an individual to a wolf or hybrid class; and the timeline on which the dog genome introgression occurred remains speculative beyond the two first generations. This suggests that the hybridisation event originated several generations in the past and most of the dog-derived alleles in such individuals have been lost, resulting in wolf genetic profile without admixture (Galaverni et al., 2017; Pilot et al., 2018). An analysis of recent data (2008–2018 periods) shows that hybridisation events in France occurred in three hotspots in the French Alps: one in Tinée, another in Maurienne and the last in Vercors (Duchamp & Queney, 2018). Wolves colonised France from neighbouring Italy as a consequence of the range expansion of the Italian population (Fabbri et al., 2007; Ciucci et al., 2009). Until recently, there were no black-coated wolves in France so it seems reasonable to hypothesise that the apparition of this phenotype in Provence is a consequence of gene flow originating from Italy; alternatively, it is possible that the hybridisation event occurred relatively recently in the French Alps. Black coat diffusion could be explained by strong positive selection and very rapid adaptations related to habitat (Caniglia et al., 2013) or immunity (Cubaynes et al., 2022), or both.

As a by-product of our study, we note that the combination of CT and use of detection dog to collect non-invasive wolf samples allow to rapidly and efficiently perform a genetic investigation of suspected hybridisation events, and conduct monitoring projects on wild and elusive species, like wolf. The combination of remote sensing and genetic analysis of non-invasive biological material is not new. In most cases the two methods are run in parallel to collect as much data as possible (Karanth et al., 2006; Mattioli et al., 2018). The analysis of scats produced by visually identifiable individuals has the potential to give full details on the structure of local packs and on the nature of breeding pairs (Canu et al., 2017). In our study, we obtained the genetic samples in two half days of field work thanks to the use of the detection dog. In a context of strong tensions related to the hot topic of hybridisation in France, speed to achieve results is fundamental. Indeed, the French Ministry of the Environment noted in a recent report that genetic results must be shared quickly; otherwise some actors will continue to confuse the public with fake news (Rapport CGED/CGAAER, 2019).

In conclusion, this study is the first to detail black-coated wolf phenotypes in France. Genetic analyzes suggest that all the sampled canids were wolves without any sign of dog admixture; or alternatively that dog genome was too diluted to be detectable. During the 2021–2022 winters all the young wolves with black phenotypes dispersed. There was no reproduction in summer 2022 in the Sirius Black wolf-pack, but a new wolf-pack including pups with black phenotypes was established during summer 2022 at 60 km of the Sainte-Baume area. The genetic monitoring of these black-coated wolves and their offspring is of scientific interest and will inform on the gene flow in French wild wolves populations. As a byproduct of our study, we conclude that a combination of CT and dog surveys to collect DNA samples is a good tool to rapidly respond to situations of urgency concerning suspicion of hybridisation events.

Ethic statements. All authors agree with the contents of the manuscript and its submission to the journal. Neither the manuscript nor any parts of its contents are currently under consideration or published in another journal. All authors disclose any potential conflict of interest with other people or organizations that could inappropriately influence their work. The Fédération Départementale des Chasseurs du Var financed the camera traps and funded genetic analyses.

Data availability. The data supporting this study's findings will be available in the Figshare Digital Repository.

Author contribution: Fabrice Roda: Conceptualization (lead), Formal analysis (lead), Field investigation (equal), Methodology (equal), Resources (supporting), Writing original draft (lead), Writing –review and editing (lead). Jean-Noël Philibert: Field investigation (equal), Methodology (equal), Resources (lead), Writing original draft (supporting).

Supplementary Material

S1: Methods
S2: Genotypes
(Supplementary Material: https://figshare.com/ndownloader/files/51849224)

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