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On the trail of medieval wolves: ancient DNA, CT-based analyses and palaeopathology of a 1000-year-old wolf cranium from the Po Valley (northern Italy)

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1 **On the trail of medieval wolves: Ancient DNA, CT-based analyses and**  
2 **paleopathology of a 1000-year-old wolf cranium from the Po Valley (northern Italy)**

3

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29

## 30 **Abstract**

31 The Middle Ages represented a crucial period for the evolutionary history of wolves (*Canis*  
32 *lupus*), marked by both significant ecosystem changes, especially through the degradation  
33 of wooded landscapes and heavy persecution, that drove this species to a dramatic  
34 demographic decline. In Europe, informative and well-documented wolf remains from the  
35 Medieval Ages are exceptionally rare and are mostly represented by teeth and postcranial  
36 elements. In this study, we describe a well-preserved wolf cranium dated to ca. 967 - 1157  
37 AD from the Po Valley (northern Italy). The specimen was analysed through a  
38 multidisciplinary approach including CT-based, ancient DNA, and palaeopathological  
39 analyses. Morphological and genetic data supported the assignment of this sample to  
40 *Canis lupus* species. CT-based analyses indicated a typical wolf-like morphology falling  
41 into the extant variability of the medium-sized subspecies *C. lupus italicus*, whereas  
42 paleopathological analyses indicated a severe periodontitis. Phylogenetic analyses  
43 showed that the Po valley wolf had a unique and never described mtDNA control region  
44 haplotype, testifying variability in the ancient Italian wolf, which has now been lost. This  
45 study provides the first comprehensive description of a wolf from the Middle Ages, adding  
46 useful information for a deeper knowledge about population dynamics, variability, and  
47 diseases of this species.

48

49 **Keywords:** *Canis lupus*; Medieval period; aDNA; Genetics; Morphology; Palaeopathology.

50

## 51 **Introduction**

52 The wolf (*Canis lupus* Linnaeus, 1758) represents one of the most iconic predators  
53 throughout human history. Its complex social behaviour, ecological adaptability and pack  
54 hunting abilities acquired over ~350 Ka of evolution (Brugal and Boudadi-Maligne 2011;  
55 Sardella et al. 2014), make the wolf one of the most successful and widespread species of  
56 the genus *Canis*. The species occurs in Eurasia and North America where it inhabits both  
57 natural (e.g., forests, grasslands, Mediterranean scrub) and anthropised (e.g., agricultural  
58 fields, urban peripheries) environments. In Europe, after centuries of persecution,  
59 abundance declines, and spatial contractions (Breitenmoser 1998), the wolf has  
60 recolonised most of its original distribution range in the Iberian, Italian and Balkan  
61 Peninsulas, as well as in several areas of central-eastern and north-western countries  
62 (Chapron et al. 2014). Paleontological studies attest a much wider wolf distribution during  
63 the Late Pleistocene (Brugal and Boudadi-Maligne 2011; Sardella et al. 2014) and  
64 Holocene (Sommer and Benecke 2005) in almost all the European continent, with a  
65 discrete presence even on large islands such as the Great Britain and Sicily (Angelici and  
66 Rossi 2018). In particular, paleontological investigations on the genus *Canis* have  
67 significantly contributed also to define several morphological (Sardella et al. 2014; Mecozzi  
68 and Bartolini Lucenti 2018; Mecozzi et al. 2017; Mecozzi et al. 2020) and chronological  
69 (Brugal and Boudadi-Maligne 2011) aspects concerning the evolution of Quaternary  
70 European wolves.

71 For example, Italian fossil records of *C. lupus*, consisting of thousands of cranial and  
72 postcranial remains spanning from the Middle Pleistocene to the Holocene, confirmed the  
73 key role played by the Italian Peninsula, due to its geographical position, in affecting Italian  
74 wolf dispersal routes and diversification dynamics during the Quaternary.

75 Recently, ancient DNA analyses performed on Late Pleistocene and Holocene remains of  
76 *C. lupus* and *C. lupus familiaris* provided new insights into ancient Italian and European  
77 wolf population dynamics and allowed to detect the oldest occurrence of domestic dogs in  
78 Italy, suggesting the presence of a possible domestication site also in the Peninsula  
79 ([Ciucani et al. 2019](#); [Angelici et al. 2019](#); [Boschin et al. 2020](#); [Koupadi et al. 2020](#)).  
80 Additionally, ancient DNA analyses on European canid samples from the nineteenth to the  
81 early twentieth centuries showed that most western European wolf populations  
82 experienced a drastic spatio-temporal reduction of their genetic diversity and that among  
83 them the Italian wolf population suffered the most severe and protracted bottleneck  
84 ([Dufresnes et al. 2018](#)).

85 Conservation genetic studies analysing extant canid samples revealed that such numerical  
86 contractions and genetic variability loss for the Italian wolf population have continued until  
87 the early 1970s ([Montana et al. 2017a,b](#)), when it reached the verge of extinction counting  
88 only approximately 100 individuals surviving in the central and southern Apennines ([Zimen  
89 and Boitani 1975](#)).

90 Since then, thanks to legal protection and socio-ecological changes ([Chapron et al. 2014](#)),  
91 Italian wolves significantly increased with 1.600 – 1.900 individuals estimated between  
92 2009 and 2013 ([Galaverni et al. 2016](#)), and more than 2.000 wolves are likely present in  
93 Italy today.

94 Interestingly, despite this wide availability of paleontological and genetic data on ancient  
95 and recent European wolf populations, morphological and genetic studies of wolves living  
96 during intermediate historical periods such as the Middle Ages are underrepresented. The  
97 few available documents from these periods are solely reports of hunting bounties and  
98 commercial exploitations, attesting the impact of human activities on wolf survival.

99 However, Middle Ages certainly represented a significant period in the evolutionary history  
100 of the wolf in Europe.

101 Though planned and rewarded hunting activities against the wolf dates back to the sixth  
102 century B.C., when Solone of Athens introduced a 1 to 5 drachma bounty for every wolf  
103 killed (Boitani 2003), persecutions on wolf populations became deeply impactful only from  
104 the beginning of the Medieval period. Since then, wolves were systematically killed  
105 throughout Europe until the end of the 1800s (Mallinson 1978; Zimen 1978) for multiple  
106 reasons, from personal and livestock protection to completely irrational motivations linked  
107 to cults and popular beliefs (Boitani 2003; Pluskowsky 2006).

108 Hunting pressures in Europe were as extensive as that the wolf was completely extirpated  
109 in the British Isles (Harmer and Shipley 1902) and Denmark (Zimen 1978; Boitani 1986)  
110 around the mid-1700s, in Switzerland and Germany before the end of the nineteenth  
111 century (Zimen 1978; Boitani 1986), whereas the last individuals were slain in Sweden and  
112 Norway in 1966 and 1973, respectively (Turi 1931; Promberger and Schröder 1993). In  
113 France, persecutions started with Charlemagne who issued two special laws between 800  
114 and 813 AD (*Capitolare de Villis* and *Capitolare Aquisgranensis*) creating specialised wolf  
115 hunters called “*louveterie*” dismissed during the French Revolution and revived in 1814  
116 (Hainard 1961). In 1883 French wolves killed reached the impressive number of 1.386 and  
117 the last individual was spotted in 1934 (Beaufort 1987). Similarly, extensive killings were  
118 perpetuated also in Spain by Carlo VI in 1404 until a few decades ago (Grande del Brio  
119 1984; Blanco et al. 1990), while in Italy wolf bounties are reported from the twelfth century  
120 to the 1950s (Boitani 2003). However, both in Spain and in Italy wolves never  
121 disappeared.

122 Despite such hunting pressures protracted for centuries, in Europe osteological remains of  
123 Medieval wolves are extremely limited (Pluskowsky 2006 and literature therein), leading to

124 a sort of paradox, because in the scientific literature more data are available for European  
125 Pleistocene wolves than for those from a few centuries ago. Among Medieval  
126 archaeozoological remains, the proportion of wild to domestic species varies according to  
127 the geographical and cultural context, but the presence of *C. lupus* is always scarce. This  
128 shortage might be due to 1) the poorly preserved nature of the samples/specimens, which  
129 often makes the distinction between wolves and dogs morphologically unfeasible; 2)  
130 Medieval remains of wild animals mainly represented by game species as ungulates,  
131 lagomorphs, and birds; 3) wolf carcasses generally ignored without gathering their bones  
132 since wolves were usually killed for defence, bounties or their pelts, but rarely exploited as  
133 a food resource.

134 This scarcity of European wolf remains in Medieval archaeological contexts is not only a  
135 question concerning the human-wolf relationship, but also results in a serious lack of  
136 biological and ecological information for a more exhaustive comprehension of the  
137 dynamics and phenomena affecting the evolution of past populations of *C. lupus*.

138 In this scenario, we morphologically and genetically analysed a perfectly preserved  
139 cranium of *C. lupus* recovered from the Po Valley (northern Italy) dated to ca. 967 – 1.157  
140 AD. which might be crucial to improve our knowledge on Middle Age wolves and their  
141 relation with the extant and fossil Italian forms. This cranium was only briefly reported in  
142 [Ciucani et al. \(2019\)](#), but in this study, we provide the first comprehensive description of  
143 the specimen based on a multidisciplinary approach, including palaeopatological, aDNA,  
144 and tomographic analyses, to advance more detailed inferences on wolf dispersal  
145 dynamics and genetic variability during the Middle Ages.

146

147 **Geographical and geological background**

148 The Po Valley ([Figure 1](#)) represents an area consisting of a layer of sediments of fluvial  
149 and glacial origin, from the Pleistocene (2,6 – 0,01 Ma) to the Holocene (from 12.000  
150 years ago to today) overlying a marine substrate that rarely emerges (Plio-Pleistocene)  
151 ([Persico et al. 2006a](#); [Bona and Corbetta 2009](#)).

152 Since the beginning of the Quaternary, glacial and interglacial phases have alternated  
153 throughout the Pleistocene characterized by general variations in the global average  
154 temperature with the consequent development or regression of polar and alpine glaciers  
155 on the Earth's surface. Between one glacial period and the next, interglacial phases  
156 alternated, during which the rise in the average temperature of the Earth caused a  
157 progressive retreat of the ice and a consequent rise in sea level. In this context, the action  
158 of the Alpine and Apennine rivers filled the Po basin, generating the plain. In particular, the  
159 more superficial layers of the plain derive from the material transported by the rivers at the  
160 end of the last glacial period (110.000 – 12.000 years ago) following the erosion of the  
161 morainic bodies previously generated by the advancement of the glacial front during the  
162 cold phase ([Persico 2021](#)).

163 The end of the Pleistocene sees a morphological arrangement of the plain quite similar to  
164 the current one, with a hydrographic network in which the main rivers are still recognizable  
165 today, with the Po as the main collector. The main traceable geomorphologies also date  
166 back to the last glacial phase. In this stretch of the plain, the Po River has a tortuous  
167 course. This morphology is due to the low slope of the plain, the intensity of the erosive  
168 and transport processes, to the geomorphology of the territory.

169 The low slope of the plain forces the river to erode the concave (external) bank and to  
170 deposit material on the convex (internal) bank. The deposited sediments make up the  
171 meander bar.

172 Following the floods, coarse sediments are deposited, which often contain fossil remains.  
173 Under normal riverbed conditions, the reduced energy of the water involves the transport  
174 of medium, fine, or very fine sediments such as gravels, sands, and silts. On the other  
175 hand, when the flow of the river increases, coinciding with seasonal flood events, erosion  
176 and transport increase, causing the deposition of larger clasts on the bars and with them  
177 also the fossil bone remains. The ease with which fossil bones are transported by river  
178 water varies according to their shape, size, and weight, and their frequency of discovery is  
179 generally related to the presumed proximity of the primary reservoir sites, never directly  
180 identified.

181 The river bar where the studied fossil was found is located downstream of the “Boschi  
182 Maria Luigia” Park, near the Coltaro di Sissa Trecasali (PR) (Figure 1). The bar has a  
183 crescent shape and is located on the right bank of the Po River, inside the meander.  
184 Sedimentologically, this bar is characterized by gravel, sand, and silt, with the second  
185 component in clear prevalence. On this beach, there are notoriously few finds, for the most  
186 ancient fossil remains, it is necessary to shift attention to the river bars upstream of the Po  
187 until reaching Cremona.

188 The ongoing research by one of the authors (D.P.) testifies to a sedimentary classification  
189 from Cremona to Casalmaggiore (CR), with a drastic decrease in the size of the clasts  
190 towards the second city, located a few kilometers downstream of the site where the wolf  
191 was found. This sedimentary characterization is linked to the distribution of paleontological  
192 finds along the river that show ancient, large, and well-preserved remains, where the  
193 granulometry of the sediments is greater, with more modern, smaller, or fragmented  
194 remains, where the sedimentology shows a reduced granulometry. This correlation can be  
195 easily explained by correlating the size of the clasts, the transport energy of the river

196 water, and the lesser or greater depth of the fossil formation under the river: superficial  
197 near Cremona and deep in nearby Casalmaggiore (CR).

198

## 199 **Materials and methods**

### 200 ***Sample collection***

201 The wolf (MSDP 348) specimen ([Figure 2](#)) analysed in the present study was recovered in  
202 September 2016 on the alluvial bar of the Po River, right bank near Coltaro (PR) but in  
203 Gussola (CR) territory, Lombardy Region (North Italy) (45° 58' 53" N; 10° 19' 49" E;  
204 altitude: 21 m a.s.l.). The area, which is well-known for its numerous paleontological  
205 Quaternary records ([Persico et al. 2006](#)), consists of a crescent-shaped meander bar  
206 (about 800 meters), located in the meander of Isola Maria Luigia, a few kilometres  
207 upstream to Casalmaggiore (CR) ([Figure 1](#)). The fossil was found in the typical  
208 allochthonous position. For this reason, there are no stratigraphic information, and the  
209 dating was possible only with a radiometric method (see below). Sample MSDP 348 is  
210 currently housed in the Carnivora fossil section of the Po River at the Paleoanthropological  
211 Museum of San Daniele Po (Cremona, Northern Italy).

212 Craniodental measurements were taken with a manual calliper to the nearest 0.1 mm.  
213 Comparative samples, used for both morphological and biometric analyses, are largely  
214 based on our personal dataset (complete list in [Tables S1, S2](#), [Figures S1, S2](#)) which  
215 includes extant *Canis lupus italicus* and Late Pleistocene *Canis lupus* from several Italian  
216 paleontological sites. For the biometric comparison we considered the cranial  
217 measurements following [von den Driesch \(1976\)](#): the total length (TL), condylobasal length  
218 (CBL), palatal length (PL), greatest breadth of the mastoid processes (GMB), greatest  
219 neurocranial breadth (GNB), greatest breadth of the palate at the level of P<sup>4</sup> (GBP), lower

220 palatal breadth at level of C<sup>1</sup> (LPB). Dental traits were also considered: length (L) and  
221 breadth (B) of I<sup>3</sup>, P<sup>2</sup>, P<sup>3</sup>, P<sup>4</sup>, M<sup>1</sup> and M<sup>2</sup>. Angular measurements were obtained using the  
222 digital tool available on Materialise Mimics 20. The age estimation was performed following  
223 [Gibson et al. \(2000\)](#) while gender determination was estimated following ([Siracusa and Lo](#)  
224 [Valvo 2004](#)).

225

### 226 **Tomography**

227 Sample MSDP 348 and a selection of extant *Canis lupus italicus* ([Tables S3, S4](#)) were  
228 acquired using a Philips Brilliance CT 64-channel scanner at M.G. Vannini Hospital  
229 (Rome). The scanning resulted in 659 slices with dimensions of 768 × 768 pixels. The  
230 slice thickness is 0,8 mm with an interslice space of 0,4 mm. The CT data were processed  
231 using Materialise Mimics 21.0 while the final 3D model was rendered with ZBrush 4R6  
232 ([Boscaini et al. 2020](#)).

233

### 234 **Radiometric dating**

235 Sampling for radiometric dating was performed in the Ancient DNA Lab of the Cultural  
236 Heritage Department of University of Bologna. Subsequently the material was analysed by  
237 the Dating and Diagnostics Center (CEDAD) of the University of Salento. Radiometric  
238 dating was performed by conventional radiocarbon method using the high-resolution mass  
239 spectrometry technique (AMS). The dating results have been corrected for the effects of  
240 isotopic fractioning both through the measurement of the  $\delta^{13}\text{C}$  term carried out directly  
241 with the accelerator and for the bottom of the measurement. Samples of known  
242 concentration of Oxalic Acid provided by NIST (National Institute of Standards and  
243 Technology) have been used for quality control of the results. For the determination of the

244 experimental error on the radiocarbon date, both the scattering of data around the average  
245 value and the statistical error deriving from the  $\delta^{14}\text{C}$  count were taken into account.  
246 Radiocarbon dating for the samples was then calibrated at the calendar age using the  
247 software OxCal Ver. 3.10 based on atmospheric data ([Reimer et al. 2013](#)).

248

### 249 ***Ancient DNA standards and analyses***

250 Molecular analyses were conducted at the ancient DNA laboratory of the University of  
251 Bologna (Ravenna Campus) following strict criteria (see [Supplemental Material](#)) in order to  
252 support the authenticity of the results and prevent possible contamination from exogenous  
253 DNA ([Cooper and Poinar 2000](#); [Gilbert et al. 2005](#); [Knapp et al. 2012](#); [Llamas et al. 2017](#)).

254 A sub-sampling for molecular analyses was performed on a tooth encased in the alveolus  
255 of the maxilla, drilling directly in the root from the tooth neck. The surface was superficially  
256 decontaminated by slight abrasion with a sterile diamond drill and then exposed to UV  
257 radiation for 30 minutes. Then, DNA was extracted from the root powder with a silica-  
258 based protocol ([Cilli et al. 2020](#)) modified from [Dabney et al. \(2013\)](#). Amplicons of 361 bp  
259 of the mitochondrial DNA were obtained by means of three overlapping fragments  
260 amplified by Polymerase Chain Reaction (PCR) using three couples of primers (See  
261 [Supplemental Material](#)).

262 Excluding primers, the three overlapping fragments covered the following nucleotide  
263 positions 15431 - 15792 of the Italian wolf mitochondrial genome (Genbank accession  
264 number KU644662). PCR reactions were conducted in triplicate, to ascertain the presence  
265 of possible miscoding lesions.

266 The purified amplicons were sequenced at the Unit for Conservation Genetics (Ozzano  
267 dell'Emilia, Bologna, Italy), Department for the Monitoring and Protection of the

268 Environment and for Biodiversity Conservation of the Italian Institute for Environmental  
269 Protection and Research (ISPRA).

270 Electropherograms were manually analysed and visually edited using the ABI software  
271 SEQSCAPE v.2.5. Consensus sequences were reconstructed by using the three  
272 replicates for each fragment, using the Italian wolf mitochondrial genome (Genbank  
273 accession number KU644662) as a reference.

274 Blastn ([Altschul et al. 1990](#)) was checked in order to analyze the sequence in the context  
275 of the ancient and modern genetic variability available in GenBank database.

276 A database of published homologous mtDNA sequences ([Table S5](#)) was constructed to  
277 analyse our data in the wider context of ancient and modern European canids. In  
278 particular, a 331-bp alignment containing ancient and modern wolf and dog sequences  
279 was used for phylogenetic reconstructions. Instead, a 278-bp alignment, containing only  
280 ancient and modern wolf sequences was used for within-species network analyses. The  
281 dimension of the two alignments was determined by the maximum length of the sequences  
282 included in the reference selected (See [Table S5](#)).

283 The software DnaSP v.5.10.01 ([Librado and Rozas 2009](#)) was used to identify identical  
284 sequences and to collapse them into unique haplotypes.

285 The 331-bp alignment was run in JModeltest2 ([Darriba et al. 2012](#)) to estimate the best  
286 nucleotide substitution model through the Akaike information criterion (AIC) and to  
287 construct in MEGA-X v.10.2.5 ([Kumar et al. 2018](#)) a Neighbor-Joining (NJ; [Saitou and Nei  
288 1987](#)) phylogenetic tree, which was rooted using a Coyote sequence (*Canis latrans*,  
289 GenBank access number FJ213927.2; [Fain et al. 2010](#)) as an outgroup and whose  
290 internode supports were obtained by 1,000 bootstrap replicates ([Felsenstein 1985](#)).  
291 Finally, the obtained tree was visualized and edited in FigTree V.1.4.4 (Rambaut 2010).

292 A Median-Joining network (MJ), based on the 278-bp alignment, was constructed by using  
293 Network v.10.2 (<https://www.fluxus-engineering.com/sharenet.htm>) with no pre- or post-  
294 processing steps.

295

## 296 **Results**

### 297 ***Radiometric dating***

298 Radiometric dating performed on a bone sample taken from the studied cranium is  
299 supported by a high probability of correctness (94%) and highlights a young age of the  
300 fossil (967 – 1.157 AD, [Figure S3](#)). The determined age is in agreement with the initial  
301 fossilization state identified.

302

### 303 ***Outer cranial anatomy***

304 Sample MSDP 348 ([Figure 2](#)) consists of an almost complete and well preserved cranium  
305 missing of both the zygomatic arches, the left I<sup>1</sup>, I<sup>2</sup>, C<sup>1</sup>, P<sup>1</sup> and the right I<sup>1</sup>-I<sup>3</sup>, C<sup>1</sup>, P<sup>1</sup>. The  
306 bone tissue appears beige in colour, compact and quite mineralised but light. The dental  
307 enamel is white-yellowish with orange traces of iron oxides. The cranial sutures appear  
308 completely fused with the exception of some portions of the fronto-parietal and  
309 presphenoidal sutures, where the welding is weaker. Both the canines are missing and  
310 their alveolar cavities are partially exposed due to the loss of bone tissue. The bone  
311 surrounding the right alveolus appears compact with sharp edges, while a marked swelling  
312 with highly vascularised newly formed bone tissue with smooth edges characterising the  
313 left alveolus. The abnormal morphologies of the latter, suggest a lesion of pathological  
314 origin rather than the result of taphonomic processes (see Paleopathology). In contrast,  
315 the fractures with smooth edges of the zygomatic arches, the strong abrasion of both the

316 zygomatic processes of the frontals, which partially expose the cavity of the frontal  
317 sinuses, as well as the partial exposure of the tooth roots of the cheek teeth and the cavity  
318 affecting the right lacrimal and palatine bones, are of taphonomic origin and probably due  
319 by a low-energy river transport (rafting). On both sides, the orbital fissure, the anterior alar  
320 foramen and the oval foramen are obliterated with very fine gravels (2-4 mm) which  
321 support the hypothesis of a long permanence in river waters.

322 The cranium MSDP 348 shows a typical wolf-like morphology that falls into the variability  
323 of extant specimens of *C. lupus italicus* (Table S1): the braincase is globose with a  
324 prominent sagittal crest distally protruding over the occipital condyles; the rostrum is robust  
325 and elongated, the frontals are quite narrow and elevated with a moderate step; the nasal  
326 bones end in correspondence of the maxillofrontal suture; the tympanic bullae are large  
327 and oval in shape in ventral view; the orbital angle is approximately 41.5°. On the distal  
328 margin of the anterior palate, the incisive foramina extend from the anterior margin of the  
329 canine alveoli to almost reach the mesial margin of the P<sup>1</sup>. The arrangement of the upper  
330 tooth row in the studied specimen is overlapping with those of the extant wolves, indeed,  
331 the C<sup>1</sup>-P<sup>2</sup> are arranged on the same axis, which changes in P<sup>3</sup>-P<sup>4</sup> and is different again in  
332 M<sup>1</sup>-M<sup>2</sup>. However, some minor differences with the extant form are noticeable. In MSDP  
333 348 the distal portion of the rostrum is slightly more flattened, the palate is larger at the  
334 level of P<sup>2</sup>-P<sup>3</sup> and the frontal bones are less wider with a more pronounced postorbital  
335 constriction (Figure 3). Taking into account the Italian crania of *C. lupus* from Middle to  
336 Late Pleistocene, is important to remark that recent studies (Sardella et al. 2014; Mecozzi  
337 et al. 2017; Mecozzi and Bartolini Lucenti 2018; Mecozzi et al. 2020) attested the presence  
338 of two distinct chronological morphotypes, consisting of “slender forms” spanning from the  
339 late Middle to early Late Pleistocene and “robust forms” from the beginning of MIS 3.  
340 According to the authors, suggesting a dispersal wave of canids from continental Europe  
341 probably adapted to cold climatic conditions. In this context, the specimen MSDP 348

342 resembles in overall morphology the robust forms from Vignone, Grotta Ladrenizza and  
343 Grotta Grimaldi with which it shares a dorsoventrally flattened rostrum and a wide and  
344 straight anterior portion of the palate without the constriction at the P<sup>2</sup>-P<sup>3</sup> observed in  
345 extant Italian wolves.

346 Also, the teeth are morphologically almost indistinguishable from those of fossil and extant  
347 specimens. In MSDP 348, the left I<sup>3</sup>, the P<sup>4</sup> and M<sup>1</sup> of both the hemiarches are quite worn,  
348 whereas the P<sup>2</sup>, P<sup>3</sup> and M<sup>2</sup> only show a slight wear on the cusp tips. The I<sup>3</sup> is separated  
349 from the I<sup>2</sup> alveolus by a short diastema, but the development of its basal cingula cannot  
350 be observed due to the advanced wear of the crown. The P<sup>1</sup> is monocusped and meso-  
351 distally elongated. The P<sup>2</sup> is meso-distally elongated with an asymmetric protocone and a  
352 weak distal cingulum. Also the P<sup>3</sup> is meso-distally elongated, but possesses a symmetric  
353 protocone, a small distal accessory cusp and a weak distal cingulum. The P<sup>4</sup> shows a  
354 weak cusp on the protocone, located slightly anteriorly than the mesial margin of the tooth  
355 and in occlusal view it appears lingually expanded. Both the paracone and metastyle are  
356 stout but not very high due to wear. The mesial cingulum is marked, whereas those labial  
357 and lingual are less developed. The M<sup>1</sup> possesses a nearly squared profile in occlusal  
358 view. It has a large paracone, considerably more developed compared to the metacone.  
359 The trigon basin is wider and deeper than the talon basin. The protocone is quite large,  
360 whereas the metaconule and the hypocone are weakly developed. The mesial and buccal  
361 cingula are pronounced. Also the M<sup>2</sup> is quite squared in shape in occlusal view, with a  
362 paracone slightly larger than the metacone. The protocone is large and rounded in profile,  
363 the basin is less deep and the hypocone cingulum is well developed. Distal and buccal  
364 cingula are marked.

365 Considering the morphology of the cheek teeth, [Mecozzi and Bartolini Lucenti \(2018\)](#)  
366 reported the presence of marked cusps and marked cingula, together to the occurrence of

367 mesial and distal accessory cusps, in the robust specimens from the Late Pleistocene of  
368 Italy. The cranium MSDP 348 shows an intermediate morphology between the slender and  
369 robust morphotypes, as the main cusps of the teeth are stout and well developed, whereas  
370 the accessory ones and cingula are less pronounced.

371

### 372 ***Inner cranial anatomy***

373 The brain and frontal sinuses of MSDP 348 show an overlapping size and morphology with  
374 those of the extant sample (Figure 3, Tables S3, S4). The frontal sinuses are large and  
375 well-developed extending from the antorbital constriction up to the fronto-parietal suture. In  
376 dorsal view, they appear slightly narrower compared to those of the extant sample. The  
377 edges of the sinuses are lobed and the surface is smooth. Laterally, they appear  
378 prominent with the typical triangular profile (Figures 3a-d).

379 The brain endocast of MSDP 348 lacks both olfactory bulbs, the telencephalon is globular  
380 in shape with marked convolutions. In dorsal view, a marked longitudinal fissure divides  
381 the cerebrum into two roughly symmetrical hemispheres characterised by the following  
382 gyri, from front to back: orbital, prorean, lateral, sigmoid, coronal, endolateral, ectolateral,  
383 suprasylvian and ectosylvian, including the respective sulci. The frontal pole cortex is  
384 antero-posteriorly elongated and the orbital gyrus is laterally expanded forming a “bump”  
385 bounded by the intraorbital sulcus (Figures 3e-h). The orbital region is characterized by the  
386 presence of three main sulci: the prorean sulcus, the intraorbital sulcus and a third sulcus  
387 considered by [Lyras and Van der Geer \(2003\)](#) as typical of *C. lupus*, *C. simensis* and *C.*  
388 *rufus*. The overall brain morphology of MSDP 348 corresponds to that of the extant wolf,  
389 except for the wider and more marked pseudosylvian fissure.

390

391 **Biometric analysis**

392 Figures 4a, S1 and Table S1 show that the specimen MSDP 348 fall within the cranial  
393 variability of the extant Italian wolf occupying the lowest dimensional ranges. The average  
394 size of MSDP 348 is between that of the Late Pleistocene specimen from Grotta Romanelli  
395 – very small and thus referred to the “slender form” – and those of larger and more robust  
396 specimens from the Late Pleistocene sites of Grotta Ladrenizza, Covoli di Velo,  
397 Romagnano and Broion (Figure 4a). Indeed, the latter occupies the highest values  
398 reached by the extant forms and are considerably larger than MSDP 348. Compared to the  
399 sample, MSDP 348 would appear to have a relatively narrow braincase (TL-GNB) and a  
400 fairly wide palate (GBP-LPB) (Figure 3, Table S1).

401 The teeth measurements indicate that MSDP 348 has a smaller I<sup>3</sup> and a larger breadth of  
402 the P<sup>4</sup> and M<sup>1</sup> compared to the extant *C. lupus italicus* (Figures 4b, S2, Table S2). All other  
403 measures fall comfortably within the tooth variability of the extant form. Biometrically the  
404 teeth of MSDP 348 resemble those of the Late Pleistocene wolves, except for the larger  
405 breadth of the M<sup>1</sup> and M<sup>2</sup> and the shorter P<sup>2</sup>.

406 The teeth of the specimen from Grotta Romanelli are the smallest of the sample, except  
407 for the length and breadth of the P<sup>2</sup> which are similar to those of the other specimens,  
408 including MSDP 348.

409

410 **Age and gender determination**

411 At first glance, the overall morphology and size of MSDP 348 clearly indicates an adult  
412 individual, but to better define the age in terms of years, we considered the welding degree  
413 of the cranial sutures and the tooth wear. According to Barone (2006), in domestic dogs  
414 the complete welding of all the cranial sutures occurs at the age of 3-4 years. In MSDP

415 348 the cranial sutures are completely welded except the fronto-parietal and  
416 presphenoidal ones where the welding is weaker suggesting an age of approximately 3  
417 years. In contrast, the I<sup>3</sup>, P<sup>4</sup> and M<sup>1</sup> are quite worn to the point that a portion of the pulp  
418 cavity is exposed on the I<sup>3</sup> and the main cusps of the P<sup>4</sup> and M<sup>1</sup> are completely blunted.  
419 Following the age estimations proposed by [Gipson \(2000\)](#) based on the tooth wear of the  
420 wolf, the upper carnassial of the Po Valley specimen corresponds to an individual between  
421 6 and 8 years of age. The discrepancy between the estimated ages from sutures and teeth  
422 is considerable and it is likely that some sutures have loosened as a result of taphonomic  
423 processes such as a long presence in water. Teeth are generally less affected by the  
424 environmental processes as the tooth enamel is harder and more resistant compared to  
425 the bones tissue, moreover in the case of MSDP 348 the wear surfaces are all located on  
426 the occlusal plane of the teeth, indicating that their origin is due to chewing activity and not  
427 to taphonomic processes. Therefore, we consider the age estimated from teeth more  
428 reliable than that obtained from the cranial sutures.

429 In the wolf skulls there is a certain sexual dimorphism that can be evaluated through the  
430 Storer index. Its formula is as follows:

$$431 \quad \quad \quad x_m - x_f / (x_m + x_f) : 2 \times 100$$

432 Where:

433  $x_m$  = average value for a character for males

434  $x_f$  = mean value of the same character for females

435 The index value is negative when the female is larger than the male, it is equal to zero  
436 when there are no differences between the sexes and it is positive when the male is larger  
437 than the female ([Siracusa and Lo Valvo 2004](#)). In the studied sample the incompleteness  
438 of the fossil did not allow the survey of all the parameters for the calculation of the index so  
439 that only some measures (condylobasal length and total length of the cranium) were  
440 compared with the values present in the literature ([Syracuse and Lo Valvo 2014](#)) ([Table 1](#)).

441 From the comparison in Table 1 it can however be estimated that MSDP 348 sample might  
442 belong to a female wolf.

443

#### 444 ***Ancient DNA analyses***

445 Molecular analyses allowed us to obtain reliable data, in accordance with the guidelines  
446 and standards applied, for all the three replicated amplifications of the three different  
447 fragments, resulting in a 361-bp mtDNA consensus sequence. Moreover, the sequence  
448 was confirmed also by the comparison with the previously published 57-bp fragment  
449 (Ciucani et al., 2019). The sequence of MSDP 348 here obtained is available on GenBank  
450 (acc. n. OL906402).

451 After blasting, the Medieval sequence returned to be unique and showed no homology  
452 with the data stored in the GenBank database. It presents one mutation of difference from  
453 the wolf haplotype named W15 by [Randi et al. \(2000\)](#) and by [Montana et al. \(2017b\)](#) and 2  
454 mutations of difference from other 4 modern wolf sequences: 3 belonging to samples  
455 collected in Italy (KU696389 and KU6446621, [Koblmüller et al. 2016](#), and KF661048,  
456 [Thalmann et al. 2013](#)) and all sharing the typical Italian haplotype W14 ([Randi et al. 2000](#);  
457 [Montana et al. 2017b](#)) and 1 belonging to a Belarusian sample (KU696390, [Koblmüller et](#)  
458 [al. 2016](#)). The subsequent haplotypes, showing more than 2 mutations of difference from  
459 the Medieval sequence we obtained, are from Bulgaria (KU696388) and Poland  
460 (KF661045).

461 The MSDP 348 sequence was also analysed against the shorter (278 bp) haplotypes  
462 retrieved in the historical (19<sup>th</sup> and 20<sup>th</sup> centuries) published dataset by [Dufresnes et al.](#)  
463 [\(2018\)](#), showing again no matches with them.

464 The best evolutionary model for the alignment of 141 canid mtDNA sequences we built  
465 was the Kimura 2-parameters model (Kimura 1980) with the rate variation among sites to  
466 Gamma distribution (G shape parameter = 1). The reconstructed NJ phylogenetic tree

467 (Figure 5), despite low supports for most nodes due to the limited length of the analysed  
468 fragment, well confirmed the distinction between the two main wolf mitochondrial  
469 haplogroups Hg1 and Hg2 proposed by Pilot et al. (2010) using partial mtDNA genomes  
470 and clearly highlighted also the distinction between the three main dog clades (A, B, C)  
471 observed by Thalmann et al. (2013) using complete mtDNA genomes. Among  
472 haplogroups, four macro-groups were also identifiable in the tree (Figure 5). Macro-groups  
473 4 and 5 include both wolf and dog (belong to canine clades A and B) haplotypes obtained  
474 from both modern and ancient samples. Macro-group 3 predominately includes modern  
475 dog haplotypes (clade A), except for four branches represented by ancient wolf  
476 haplotypes. Macro-group 1 hosts almost exclusively dog haplotypes belonging to clade C,  
477 whereas macro-group 2, which includes the sequence obtained from sample MSDP 348,  
478 is exclusively composed of ancient and modern wolf haplotypes.

479 In particular, sample MSDP 348 is clearly located in a specific wolf clade, which includes  
480 only mtDNA sequences from the Mediterranean Basin belonging to haplogroup 2 (Pilot et  
481 al. 2010). In fact, MSDP 348 haplotype is clearly connected with W15 and W14  
482 haplotypes, differing only one and two mutations from them, respectively.

483 Network analyses (Figure 6) confirmed the placement of sample MSDP 348 in the wolf  
484 haplogroup 2, and in this shorter alignment, it matches with the W15 wolf haplotype (Randi  
485 et al. 2000). Samples included in the shorter alignment showed an almost complete  
486 turnover in the wolf haplotypic composition in the European area, moving from Late  
487 Pleistocene to the current era. Indeed, all but one haplotype resulted to be different  
488 between the two considered periods: Late Pleistocene – Holocene (>2.000 yrs BP) and  
489 Holocene (<2.000 yrs BP) – present.

490

491 ***Paleopathology***

492 Among the various diseases affecting the oral cavity of canids, periodontitis is one of the  
493 most common (Strillou et al. 2010; Iurino et al. 2015). Caused by specific microorganisms  
494 which lead to the formation of periodontal pockets and the loss of alveolar bone tissue, it is  
495 often the result of pre-existing gingivitis, gingival lesions or accumulation of supragingival  
496 plaque and calculus (Brown et al. 1989; Pejčić et al. 2007; Strillou et al. 2010). In canids, a  
497 chronic inflammation of the oral cavity provokes an increase of the gingival blood flow from  
498 200 to 400% with a consequent enlargement of the vascular pores in the maxillary and  
499 dentary bones (Kaplan et al. 1982; Hock and Kim 1987; Zoellner 1990), as can be  
500 observed on the left canine alveolus of MSDP 348 (Figure 5). The protrusion of bacterial  
501 infection may have triggered the periodontitis in MSDP 348 by forming gingival pockets  
502 and causing the total loss of the canine and the retraction of the bone tissue. The most  
503 serious lesions can be noticed on the outer edge of the alveolus and within the alveolar  
504 cavity. Here the bone tissue appears swollen and highly vascularized with a consistent  
505 missing portion of the maxillary bone forming a large hole (max diameter of 12.2 mm) that  
506 connect the alveolar cavity with the nasal one (Figure 7). The smooth edges of the cavity  
507 and its sheltered position exclude a taphonomic origin of the lesion. Additional small  
508 cavities connect the alveolus of the left canine with that of P<sup>1</sup>, reaching in part the anterior  
509 root of the P<sup>2</sup>.

510 The prevalence and severity of periodontitis in canids increase mainly with age, although  
511 multiple factors such as the nature of the infection, previous lesions, genetics and diet may  
512 favour the onset of this disease and influence its pathological course (Strillou et al. 2010).  
513 Measurements carried out on spontaneous or experimental cases demonstrated that 4–6  
514 months are needed to provoke a root exposure of 5 mm (Strillou et al. 2010; Kinoshita et  
515 al. 1997; Pallua and Suschek 2011). Iurino et al (2013) documented a case of severe  
516 periodontitis in a Pleistocene *Cuon alpinus* from the San Sidero locality (southern Italy)  
517 inferring a pathological course of about 6 months. In MSDP 348, the loss of the alveolar

518 bone has exposed at least one half of the canine root (12.5 mm), thus indicating a  
519 protracted periodontitis for a period of about 12 months. Although the severity of the  
520 lesions has contributed to debilitating the individual, probably compromising its chewing  
521 and predatory abilities, however, on the basis of the available data it is not possible to  
522 establish whether its death was directly related to this pathology.

523

## 524 **Discussion and conclusions**

525 The nearly complete cranium MSDP 348 recovered from the alluvial bar of the Po River  
526 revealed an age of ca. 967-1157 AD by radiometric dating. This chronology makes the  
527 sample of particular relevance for the study of past wolf populations as the  
528 archaeozoological record of well-preserved remains of *C. lupus* are extremely rare,  
529 especially those of the Middle Ages.

530 Biometric and CT-based analyses indicate that the specimen MSDP 348 falls within the  
531 cranial variability of the extant *Canis lupus italicus* occupying the lowest dimensional  
532 ranges. The tooth wear allows to refer the cranium to an adult individual between 6 and 8  
533 years, while gender determination, performed following [Syracuse and Lo Valvo \(2014\)](#),  
534 suggests that it could be a female individual. This specimen shows clear evidence of a  
535 severe periodontitis which caused the complete loss of the left canine producing a large  
536 hole connecting the alveolus with the nasal cavity. This condition probably severely  
537 debilitated the subject, although it is not possible to establish whether the death was a  
538 consequence of this disease.

539 Biometric and CT-based findings were clearly supported also by molecular investigations,  
540 which were performed to study this sample through a multidisciplinary approach especially  
541 to analyse its gene pool into the context of modern and ancient Eurasian wolf genetic  
542 variability. Ancient DNA methodologies and strict reliability criteria here applied clearly  
543 supported the authenticity of the data obtained. The strategy of analysis used in this study

544 based on the amplification of three short and adjacent fragments, successively  
545 concatenated during bioinformatic analyses, allowed us to obtain a longer consensus  
546 mtDNA sequence compared to the fragments analysed in similar aDNA studies based on  
547 samples collected from the same latitudes ([Dufresnes et al. 2018](#); [Ciucani et al. 2019](#)).  
548 Notably, the sequence we obtained in this study resulted to be even ~300 bp longer  
549 respect also to the sequence previously obtained for the same sample by [Ciucani et al.](#)  
550 [\(2019\)](#) and that allowed us to compare the results to a much wide database of ancient and  
551 modern wolves and dogs.

552 Our phylogenetic analyses unambiguously agree with previous molecular modern and  
553 ancient studies, resulting in similar topologies for both networks and trees ([Montana et al.](#)  
554 [2017a](#); [Ciucani et al. 2019](#)).

555 Molecular comparisons showed that the MSDP 348 sequence has a unique haplotype, not  
556 recorded in the ancient (for the data available so far) nor in the modern wolf variability.

557 Interestingly, phylogenetic analyses placed mtDNA gene pool of sample MSDP 348 within  
558 the genetic variability of modern wolves, clearly distinct from that of dogs. In particular, it  
559 falls in the wolf haplogroup 2, the oldest one, which today has been largely replaced by  
560 haplogroup 1 that arrived in Europe at least 2.700 – 1.200 years ago ([Pilot et al. 2010](#)).

561 Phylogenetic analyses also showed that sample MSDP 348 haplotype is placed near the  
562 Greek haplotype named W15 ([Randi et al. 2000](#)), from which it displays only one mutation  
563 of difference. However, this difference did not emerge in the comparison based on the  
564 shorter fragment previously obtained by [Ciucani et al. \(2019\)](#), because the distinctive  
565 mutation is placed in the portion of the mitochondrial DNA that was not previously  
566 investigated, highlighting the importance of considering wide amplicons even in aDNA  
567 analyses and the success of the strategy here adopted in obtaining a longer mtDNA  
568 sequence compared to previous aDNA studies.

569 This sequence represents part of the ancient wolf variability today lost, as a result of the  
570 negative impact of overkilling and anthropic persecutions perpetrated in Medieval times  
571 and, for western Europe, in particular in the last 150 years. The study of modern and  
572 ancient samples allows us to better comprehend the dynamics of wolf populations and the  
573 alternation of haplogroups in the European areas. This didn't occur in Italy, where only  
574 haplogroup 2 is still present.

575 This study highlights the fragmentation and the few availabilities of archaeozoological and  
576 paleogenomics data from the Medieval period, a period of dark centuries for the wildlife  
577 and its management.

578 Despite *Canis lupus* is undoubtedly one of the most iconic and extensively studied  
579 predators of all times, in Europe informative osteological remains of Medieval wolves are  
580 extremely rare, limiting our comprehension of the dynamics and phenomena affecting the  
581 evolution of past populations of *C. lupus*. The perfectly preserved cranium (MSDP 348)  
582 dated to ca. 967-1.157 AD from the Po Valley (northern Italy) is here assigned to an adult  
583 *C. lupus*. Tomographic and biometric analyses of both outer and inner cranial anatomy,  
584 indicate a 6 – 8 years old female affected by a severe periodontitis, with size and  
585 morphology similar to those of the smaller specimens of extant *C. lupus italicus*. Genetic  
586 analysis reveals that the haplotype of MSDP 348 is related to the Greek haplotype W15,  
587 but was never recorded before, representing a portion of ancient wolf variability today lost.  
588 This study provides the most comprehensive description of a *C. lupus* from the Middle  
589 Ages in Italy, and underlines how archaeozoological samples represent an essential  
590 source of information to understand the dynamics, diversity and distribution of past and  
591 present-day wolves.

592

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602

#### 603 **Disclosure statement**

604 No potential competing interest was reported by the authors.

605

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778

779 **Figure captions**

780 **Figure 1.** Location of the site and contextual photo of the specimen at the moment of the  
781 discovery.

782 **Figure 2.** *Canis lupus* MSDP 348 from Po Valley (northern Italy), in left lateral (a), right  
783 lateral (b), dorsal (c), ventral (d), frontal (e) and caudal (f) views. Scale bar: 50 mm.

784 **Figure 3.** Comparison of the inner cranial anatomy. *Canis lupus* MSDP 348 (a-d), in lateral  
785 (a) and dorsal (b) views, brain endocast in dorsal (c) and lateral (d) views. Extant *Canis*  
786 *lupus italicus* (e-h), in lateral (e) and dorsal (f) views, brain endocast in dorsal (g) and  
787 lateral (h) views. Black arrows indicate the breadth of the palate and frontal sinuses. Scale  
788 bars: 50 mm.

789 **Figure 4.** Biometric comparison between *Canis lupus* MSDP 348 and a selected sample of  
790 fossil and extant *Canis lupus* (for the complete list of measurements see [Figure S1](#), [Tables](#)  
791 [S1](#), [S2](#)). Cranium (a), condylobasal length (**CBL**), greatest neurocranial breadth (**GNB**),  
792 greatest palatal breadth (**GBP**) and lower palatal breadth (**LPB**). Upper fourth premolar  
793 (b), length (**L**) and breadth (**B**). Measurements in mm.

794 **Figure 5.** Neighbour-joining (NJ) phylogenetic tree computed in MEGA using a Kimura 2-  
795 parameters model. The NJ tree was obtained aligning 331 base pairs of 141 canid mtDNA  
796 sequences (wolf haplotypes are represented by orange dots and dog haplotypes by green  
797 dots) and was rooted using a coyote (*Canis latrans*) homologue sequence. Bootstrap  
798 percentages  $\geq 50\%$  after 1.000 replicates are shown by asterisks. The two main wolf  
799 haplogroups (HG1 and HG2) are indicated by yellow and light blue colors. Numbers from 1  
800 to 4 show the main clades of the tree. Full and empty circles distinguish extant and extinct  
801 haplotypes. In the upper right box, a zoom on tree's branches close to MSDP384 is  
802 represented.

803 **Figure 6.** Median joining network. (a) Late Pleistocene and Holocene (>2.000 yrs BP) wolf  
804 haplotypes. (b) Holocene (<2.000 yrs BP) and current wolf haplotypes. Haplogroups (HG)

805 1 and 2 are highlighted by the two shaded areas. Black dots represent median vectors.  
806 Samples included in the network refer to the 278-bp alignment and are listed in [Table S5](#).  
807 **Figure 7.** Periodontitis affecting the left canine alveolus of *Canis lupus* MSDP 348. Left  
808 lateral photo (a) and inner view of the alveolus from the 3D model. The white asterisks  
809 indicate the hole connecting the base of the alveolus with the nasal cavity. Scale bar: 2  
810 cm.

811

#### 812 **Table caption**

813 **Table 1.** The values for male and female wolves referred to *Canis lupus italicus* ([Siracusa](#)  
814 [and Lo Valvo 2014](#)).