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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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Published Version:

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) / Iurino D.A.; Cilli E.; Caniglia R.; Fabbri E.; Mecozzi B.; Ciucani M.M.; Sardella R.; Persico D.. - In: HISTORICAL BIOLOGY. - ISSN 0891-2963. - ELETTRONICO. - 35:6(2023), pp. 976-987. [10.1080/08912963.2022.2071710]

This version is available at: https://hdl.handle.net/11585/892344 since: 2023-06-27

Published:

DOI: http://doi.org/10.1080/08912963.2022.2071710

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Iurino, Dawid A., Elisabetta Cilli, Romolo Caniglia, Elena Fabbri, Beniamino Mecozzi, Marta Maria Ciucani, Raffaele Sardella, and Davide Persico. 2023. "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)." *Historical Biology* 35 (6): 976–87.

The final published version is available online at: https://doi.org/10.1080/08912963.2022.2071710.

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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)
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30 Abstract

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

48

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

50

51 Introduction

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

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

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

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

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

Interestingly, despite this wide availability of paleontological and genetic data on ancient and recent European wolf populations, morphological and genetic studies of wolves living during intermediate historical periods such as the Middle Ages are underrepresented. The few available documents from these periods are solely reports of hunting bounties and commercial exploitations, attesting the impact of human activities on wolf survival. 99 However, Middle Ages certainly represented a significant period in the evolutionary history100 of the wolf in Europe.

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

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

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

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

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

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

146

147 Geographical and geological background

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

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

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

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

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

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

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

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

198

199 Materials and methods

200 Sample collection

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

Craniodental measurements were taken with a manual calliper to the nearest 0.1 mm. 212 Comparative samples, used for both morphological and biometric analyses, are largely 213 214 based on our personal dataset (complete list in Tables S1, S2, Figures S1, S2) which includes extant Canis lupus italicus and Late Pleistocene Canis lupus from several Italian 215 paleontological sites. For the biometric comparison we considered the cranial 216 measurements following von den Driesch (1976): the total length (TL), condylobasal length 217 (CBL), palatal length (PL), greatest breadth of the mastoid processes (GMB), greatest 218 neurocranial breadth (GNB), greatest breadth of the palate at the level of P⁴ (GBP), lower 219

palatal breadth at level of C¹ (LPB). Dental traits were also considered: length (L) and
breadth (B) of I³, P², P³, P⁴, M¹ and M². Angular measurements were obtained using the
digital tool available on Materialise Mimics 20. The age estimation was performed following
Gibson et al. (2000) while gender determination was estimated following (Siracusa and Lo
Valvo 2004).

225

226 **Tomography**

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

233

234 Radiometric dating

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

experimental error on the radiocarbon date, both the scattering of data around the average value and the statistical error deriving from the δ 14C count were taken into account. Radiocarbon dating for the samples was then calibrated at the calendar age using the software OxCal Ver. 3.10 based on atmospheric data (Reimer et al. 2013).

248

249 Ancient DNA standards and analyses

Molecular analyses were conducted at the ancient DNA laboratory of the University of Bologna (Ravenna Campus) following strict criteria (see Supplemental Material) in order to 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 of the maxilla, drilling directly in the root from the tooth neck. The surface was superficially 255 decontaminated by slight abrasion with a sterile diamond drill and then exposed to UV 256 radiation for 30 minutes. Then, DNA was extracted from the root powder with a silica-257 based protocol (Cilli et al. 2020) modified from Dabney et al. (2013). Amplicons of 361 bp 258 of the mitochondrial DNA were obtained by means of three overlapping fragments 259 amplified by Polymerase Chain Reaction (PCR) using three couples of primers (See 260 Supplemental Material). 261

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

The purified amplicons were sequenced at the Unit for Conservation Genetics (Ozzano 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).

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

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

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

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

The 331-bp alignment was run in JModeltest2 (Darriba et al. 2012) to estimate the best nucleotide substitution model through the Akaike information criterion (AIC) and to construct in MEGA-X v.10.2.5 (Kumar et al. 2018) a Neighbor-Joining (NJ; Saitou and Nei 1987) phylogenetic tree, which was rooted using a Coyote sequence (*Canis latrans*, GenBank access number FJ213927.2; Fain et al. 2010) as an outgroup and whose internode supports were obtained by 1,000 bootstrap replicates (Felsenstein 1985). Finally, the obtained tree was visualized and edited in FigTree V.1.4.4 (Rambaut 2010). A Median-Joining network (MJ), based on the 278-bp alignment, was constructed by using Network v.10.2 (https://www.fluxus-engineering.com/sharenet.htm) with no pre- or postprocessing steps.

295

296 **Results**

297 Radiometric dating

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

302

303 Outer cranial anatomy

Sample MSDP 348 (Figure 2) consists of an almost complete and well preserved cranium 304 missing of both the zygomatic arches, the left I¹, I², C¹, P¹ and the right I¹-I³, C¹, P¹. The 305 bone tissue appears beige in colour, compact and guite mineralised but light. The dental 306 enamel is white-yellowish with orange traces of iron oxides. The cranial sutures appear 307 completely fused with the exception of some portions of the fronto-parietal and 308 309 presphenoidal sutures, where the welding is weaker. Both the canines are missing and their alveolar cavities are partially exposed due to the loss of bone tissue. The bone 310 surrounding the right alveolus appears compact with sharp edges, while a marked swelling 311 with highly vascularised newly formed bone tissue with smooth edges characterising the 312 left alveolus. The abnormal morphologies of the latter, suggest a lesion of pathological 313 origin rather than the result of taphonomic processes (see Paleopathology). In contrast, 314 the fractures with smooth edges of the zygomatic arches, the strong abrasion of both the 315

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 of extant specimens of C. lupus italicus (Table S1): the braincase is globose with a 323 prominent sagittal crest distally protruding over the occipital condyles; the rostrum is robust 324 and elongated, the frontals are quite narrow and elevated with a moderate step; the nasal 325 bones end in correspondence of the maxillofrontal suture; the tympanic bullae are large 326 and oval in shape in ventral view; the orbital angle is approximately 41.5°. On the distal 327 margin of the anterior palate, the incisive foramina extend from the anterior margin of the 328 canine alveoli to almost reach the mesial margin of the P¹. The arrangement of the upper 329 330 tooth row in the studied specimen is overlapping with those of the extant wolves, indeed, the C¹-P² are arranged on the same axis, which changes in P³-P⁴ and is different again in 331 M¹-M². However, some minor differences with the extant form are noticeable. In MSDP 332 348 the distal portion of the rostrum is slightly more flattened, the palate is larger at the 333 level of P²-P³ and the frontal bones are less wider with a more pronounced postorbital 334 constriction (Figure 3). Taking into account the Italian crania of C. lupus from Middle to 335 Late Pleistocene, is important to remark that recent studies (Sardella et al. 2014; Mecozzi 336 et al. 2017; Mecozzi and Bartolini Lucenti 2018; Mecozzi et al. 2020) attested the presence 337 338 of two distinct chronological morphotypes, consisting of "slender forms" spanning from the late Middle to early Late Pleistocene and "robust forms" from the beginning of MIS 3. 339 According to the authors, suggesting a dispersal wave of canids from continental Europe 340 341 probably adapted to cold climatic conditions. In this context, the specimen MSDP 348

resembles in overall morphology the robust forms from Vignone, Grotta Ladrenizza and Grotta Grimaldi with which it shares a dorsoventrally flattened rostrum and a wide and straight anterior portion of the palate without the constriction at the P²-P³ observed in extant Italian wolves.

Also, the teeth are morphologically almost indistinguishable from those of fossil and extant 346 specimens. In MSDP 348, the left I³, the P⁴ and M¹ of both the hemiarches are quite worn, 347 whereas the P², P³ and M² only show a slight wear on the cusp tips. The I³ is separated 348 from the I² alveolus by a short diastema, but the development of its basal cingula cannot 349 be observed due to the advanced wear of the crown. The P¹ is monocupsed and meso-350 351 distally elongated. The P² is meso-distally elongated with an asymmetric protocone and a weak distal cingulum. Also the P³ is meso-distally elongated, but possesses a symmetric 352 protocone, a small distal accessory cusp and a weak distal cingulum. The P⁴ shows a 353 weak cusp on the protocone, located slightly anteriorly than the mesial margin of the tooth 354 and in occlusal view it appears lingually expanded. Both the paracone and metastyle are 355 stout but not very high due to wear. The mesial cingulum is marked, whereas those labial 356 and lingual are less developed. The M¹ possesses a nearly squared profile in occlusal 357 view. It has a large paracone, considerably more developed compared to the metacone. 358 The trigon basin is wider and deeper than the talon basin. The protocone is guite large, 359 whereas the metaconule and the hypocone are weakly developed. The mesial and buccal 360 cingula are pronounced. Also the M² is guite squared in shape in occlusal view, with a 361 paracone slightly larger than the metacone. The protocone is large and rounded in profile, 362 the basin is less deep and the hypocone cingulum is well developed. Distal and buccal 363 364 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

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

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

390

391 Biometric analysis

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

The teeth measurements indicate that MSDP 348 has a smaller I^3 and a larger breadth of the P⁴ and M¹ compared to the extant *C. lupus italicus* (Figures 4b, S2, Table S2). All other measures fall comfortably within the tooth variability of the extant form. Biometrically the teeth of MSDP 348 resemble those of the Late Pleistocene wolves, except for the larger breadth of the M¹ and M² and the shorter P².

The teeth of the specimen from Grotta Romanelli are the smallest of the sample, except for the length and breadth of the P^2 which are similar to those of the other specimens, including MSDP 348.

409

410 Age and gender determination

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

348 the cranial sutures are completely welded except the fronto-parietal and 415 presphenoidal ones where the welding is weaker suggesting an age of approximately 3 416 years. In contrast, the I³, P⁴ and M¹ are quite worn to the point that a portion of the pulp 417 cavity is exposed on the I³ and the main cusps of the P⁴ and M¹ are completely blunted. 418 Following the age estimations proposed by Gipson (2000) based on the tooth wear of the 419 wolf, the upper carnassial of the Po Valley specimen corresponds to an individual between 420 6 and 8 years of age. The discrepancy between the estimated ages from sutures and teeth 421 is considerable and it is likely that some sutures have loosened as a result of taphonomic 422 processes such as a long presence in water. Teeth are generally less affected by the 423 424 environmental processes as the tooth enamel is harder and more resistant compared to the bones tissue, moreover in the case of MSDP 348 the wear surfaces are all located on 425 the occlusal plane of the teeth, indicating that their origin is due to chewing activity and not 426 to taphonomic processes. Therefore, we consider the age estimated from teeth more 427 reliable than that obtained from the cranial sutures. 428

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

431

 $xm - xf / (xm + xf) : 2 \times 100$

432 Where:

433 xm = average value for a character for males

434 xf = mean value of the same character for females

The index value is negative when the female is larger than the male, it is equal to zero when there are no differences between the sexes and it is positive when the male is larger than the female (Siracusa and Lo Valvo 2004). In the studied sample the incompleteness of the fossil did not allow the survey of all the parameters for the calculation of the index so that only some measures (condylobasal length and total length of the cranium) were compared with the values present in the literature (Syracuse and Lo Valvo 2014) (Table 1). From the comparison in Table 1 it can however be estimated that MSDP 348 sample mightbelong to a female wolf.

443

444 Ancient DNA analyses

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

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

The MSDP 348 sequence was also analysed against the shorter (278 bp) haplotypes retrieved in the historical (19th and 20th centuries) published dataset by Dufresnes et al. (2018), showing again no matches with them.

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

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

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

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

490

491 *Paleopathology*

Among the various diseases affecting the oral cavity of canids, periodontitis is one of the 492 most common (Strillou et al. 2010; Iurino et al. 2015). Caused by specific microorganisms 493 which lead to the formation of periodontal pockets and the loss of alveolar bone tissue, it is 494 often the result of pre-existing gingivitis, gingival lesions or accumulation of supragingival 495 plaque and calculus (Brown et al. 1989; Pejčić et al. 2007; Strillou et al. 2010). In canids, a 496 chronic inflammation of the oral cavity provokes an increase of the gingival blood flow from 497 200 to 400% with a consequent enlargement of the vascular pores in the maxillary and 498 dentary bones (Kaplan et al. 1982; Hock and Kim 1987; Zoellner 1990), as can be 499 observed on the left canine alveolus of MSDP 348 (Figure 5). The protraction of bacterial 500 501 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 serious lesions can be noticed on the outer edge of the alveolus and within the alveolar 503 cavity. Here the bone tissue appears swollen and highly vascularized with a consistent 504 missing portion of the maxillary bone forming a large hole (max diameter of 12.2 mm) that 505 connect the alveolar cavity with the nasal one (Figure 7). The smooth edges of the cavity 506 and its sheltered position exclude a taphonomic origin of the lesion. Additional small 507 cavities connect the alveolus of the left canine with that of P¹, reaching in part the anterior 508 root of the P^2 . 509

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

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

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

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

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

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.

Interestingly, phylogenetic analyses placed mtDNA gene pool of sample MSDP 348 within the genetic variability of modern wolves, clearly distinct from that of dogs. In particular, it falls in the wolf haplogroup 2, the oldest one, which today has been largely replaced by 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 Greek haplotype named W15 (Randi et al. 2000), from which it displays only one mutation 562 of difference. However, this difference did not emerge in the comparison based on the 563 shorter fragment previously obtained by Ciucani et al. (2019), because the distinctive 564 mutation is placed in the portion of the mitochondrial DNA that was not previously 565 investigated, highlighting the importance of considering wide amplicons even in aDNA 566 analyses and the success of the strategy here adopted in obtaining a longer mtDNA 567 sequence compared to previous aDNA studies. 568

This sequence represents part of the ancient wolf variability today lost, as a result of the negative impact of overkilling and anthropic persecutions perpetrated in Medieval times and, for western Europe, in particular in the last 150 years. The study of modern and ancient samples allows us to better comprehend the dynamics of wolf populations and the alternation of haplogroups in the European areas. This didn't occur in Italy, where only 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 on of the most iconic and extensively studied predators of all times, in Europe informative osteological remains of Medieval wolves are 579 extremely rare, limiting our comprehension of the dynamics and phenomena affecting the 580 581 evolution of past populations of *C. lupus*. The perfectly preserved cranium (MSDP 348) dated to ca. 967-1.157 AD from the Po Valley (northern Italy) is here assigned to an adult 582 C. lupus. Tomographic and biometric analyses of both outer and inner cranial anatomy, 583 indicate a 6 - 8 years old female affected by a severe periodontitis, with size and 584 morphology similar to those of the smaller specimens of extant C. lupus italicus. Genetic 585 586 analysis reveals that the haplotype of MSDP 348 is related to the Greek haplotype W15, but was never recorded before, representing a portion of ancient wolf variability today lost. 587 This study provides the most comprehensive description of a C. lupus from the Middle 588 Ages in Italy, and underlines how archaeozoological samples represent an essential 589 source of information to understand the dynamics, diversity and distribution of past and 590 591 present-day wolves.

592

593 Acknowledgments

We would like to thank M. Danti of the 'M.G. Vannini' Hospital of Rome for making the CT 594 scanning. We also thank D. Luiselli, F. Fontani and A. Latorre of the Ancient DNA 595 laboratory of the University of Bologna for their support and advice, N. Mucci (ISPRA) for 596 logistic support in the genetic analysis and E. Velli (ISPRA) for laboratory assistance. We 597 are grateful to Adam J. Andrews for English revision and useful comments. This work has 598 benefited from the equipment and the framework of the COMP-HUB Initiative, funded by 599 the 'Departments of Excellence' program of the Italian Ministry for Education, University 600 and Research (MIUR, 2018-2022). 601

602

603 **Disclosure statement**

No potential competing interest was reported by the authors.

605

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779 Figure captions

Figure 1. Location of the site and contextual photo of the specimen at the moment of thediscovery.

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

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

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

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

Figure 6. Median joining network. (a) Late Pleistocene and Holocene (>2.000 yrs BP) wolf haplotypes. (b) Holocene (<2.000 yrs BP) and current wolf haplotypes. Haplogroups (HG) 1 and 2 are highlighted by the two shaded areas. Black dots represent median vectors.
Samples included in the network refer to the 278-bp alignment and are listed in Table S5.
Figure 7. Periodontitis affecting the left canine alveolus of *Canis lupus* MSDP 348. Left
lateral photo (a) and inner view of the alveolus from the 3D model. The white asterisks
indicate the hole connecting the base of the alveolus with the nasal cavity. Scale bar: 2
cm.

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812 Table caption

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