Article

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Keywords: *Demodex*; *Canis lupus*; wolf; qPCR; *18S rRNA* gene; *16S rRNA* gene

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First Detection of Human- and Dog-Associated *Demodex* Mites (Acari, Arachnida) in Southern European Wolves (*Canis lupus*)

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Abstract

Demodex mites are common commensals of mammalian skin, but under certain conditions, they can cause severe skin diseases. This study analyzed the presence, diversity, and phylogenetic relationships of *Demodex* species in two wolf subspecies from southern Europe to determine whether species-level differences exist between wild and domestic canids after thousands of years of divergence. A total of 1,400 hair samples from 140 wolves were analyzed using qPCR targeting mitochondrial *16S rRNA* and nuclear *18S rRNA* genes. Overall, 37% of wolves were positive for *Demodex* DNA, with a higher prevalence in Italian (46%) than in Iberian (36%) wolves. The lip and chin areas were the most reliable sampling sites. Four *Demodex* species were identified in wolves: *D. injai* and *D. canis* (associated with dogs), and *D. folliculorum* and *D. brevis* (associated with humans). Co-infestations involving multiple *Demodex* species were recorded for the first time in wild canids. These results challenge the long-held belief of strict host specificity in *Demodex* mites. The discovery of *Demodex* species associated with both humans and dogs in wolves supports the idea that host-switching and ecological interactions have occurred throughout the evolution of canids and humans. Such cross-species transfers may have taken place during the early domestication of dogs. However, given the isolated history of the two southern wolf populations, it is more probable that these findings result from recent interspecific transmission events, likely facilitated by ecological overlap with domestic animals and human environments. Future genomic studies will be essential for clarifying the evolutionary relationships within the genus *Demodex* and its host associations.

Keywords: *Demodex; Canis lupus*; wolf; qPCR; *18S rRNA* gene; *16S rRNA* gene

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1. Introduction

Demodex mites are considered normal inhabitants in mammalian skin [1–3]. Currently, more than 80 species of *Demodex* spp. have been described, genetically and/or morphologically, in more than 80 wild and domestic mammals, including humans, dogs, cats, deer, ferrets, otters, and mice [2,4–14]. Most mammals harbor *Demodex* mites on the skin without developing lesions or any other clinical signs [1,15]. Multiple pieces of evidence and studies indicate that the immune system controls *Demodex* populations on the skin [1,3,16]. Although *Demodex* mites are considered commensals in

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small numbers, when they proliferate, they may cause severe dermatitis, for instance, rosacea in humans or demodicosis in dogs and cats [17–19]. In almost all cases, proliferation results from a compromised immune system, as occurs in transgenic mice [16,20] or in dogs or humans receiving immunosuppressive medical treatment [3,21,22].

Genetic sequencing has proven highly effective for detecting and identifying *Demodex* mites in both healthy and diseased mammals [5,8,10,15]. Because of the sensitivity and specificity of genetic techniques, mites can be detected where non-molecular methods, such as microscopy or trichoscopy, fail to yield results [23,24]. *Demodex* mites have been detected, identified, and classified in humans, dogs, cats, mice, and ferrets using different genetic approaches such as the mitochondrial *16S rRNA* gene region [5,7,8,25], and the nuclear *18S rRNA* gene region [2,10,26,27]. Ravera et al. (2013) [15], using a fragment of the *chitin synthase* gene, concluded that *Demodex* DNA can be detected in the skin of any dog if 20 or more skin sites are sampled. In wolves, *Demodex* mites have been detected in fecal samples [28–30]. Since *Demodex* spp. are hair follicle mites, their presence in feces could be explained by accidental ingestion during grooming or from the prey itself. Nevertheless, it remains unknown which *Demodex* species are present in wolves' skin.

Research on *Demodex* mites is relevant not only to medicine but also to evolutionary biology. Their parasitic association with mammals may date back approximately 220 million years, coinciding with the emergence of hair follicles [9,31]. Roughly 100 million years ago, the clade Boreoeutheria diverged into two sister lineages: Euarchontoglires, which includes primates, and Laurasiatheria, which includes canids [32]. *Demodex* mites have been documented, both morphologically and genetically, in species from both groups, suggesting either a long-term stability of the hair follicle niche or a high capacity for mite transmission across and within placental mammals [5,6,8,10,27,33,34]. The genus *Demodex* therefore represents an exceptional case of parasitic parallelism [2,9,15,35].

The domestic dog originated from the gray wolf (*Canis lupus*) and is associated with Neolithic humans on most continents [36–38]. Axelsson et al. (2012) [39] suggest that dogs descended from scavenger wolves adapted to a starch-rich diet during the dawn of the agricultural revolution. However, it remains unclear where domestication occurred and whether it occurred once or multiple times worldwide. Archaeological records suggest different origins and domestication times: fossilized early dog remains have been found in Belgium (36,000 years ago; [40]), eastern Siberia (33,500 years ago; [41]) , and northern Israel (12,000 years ago; [42]). Nevertheless, specimens older than 14,000 years do not appear related to modern dogs [41]. Genetic studies also yield different results. Autosomal single nucleotide polymorphism (SNP) data indicate contributions from Middle Eastern and European wolves [43], while shotgun sequencing data suggest an origin in Eurasia, possibly southern Asia [44]. Wang et al. (2015) [45] propose that domestication began around 33,000 years ago in East Asia, followed by migrations toward the Middle East and Europe. More recently, Bergström et al. (2022) [46] found that modern dogs are genetically closer to ancient wolves from eastern Eurasia than to those from western Eurasia. However, Freedman et al. (2014) [47] found that no extant wolf lineage is more closely related to dogs, implying that an extinct wolf lineage was their ancestor. In any case, dog domestication can be traced back at least 15,000 years.

The gray wolf (*Canis lupus*) is listed on the IUCN Red List (2018) as “Least Concern” and is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (except for the populations of Bhutan, India, Nepal, and Pakistan, which are listed in Appendix I). Currently, five subspecies of *Canis lupus* are recognized in North America and seven in Eurasia. This classification is primarily based on genetic and morphological differences among populations, reflecting adaptations to distinct climates, latitudes, or habitats [48–51]. In Western Europe, the differentiation between the subspecies *C. l. signatus of* the Iberian Peninsula and *C. l. italicus* of the Italian Peninsula is the result of prolonged geographic isolation, which continues to this day [52–55].

Our study aims to determine whether there are species-level differences in *Demodex* mites between wild and domestic canids after this extensive period of divergence. For this purpose, we analyzed 1,400 hair samples from 140 wolves belonging to the two southern European subspecies, using two gene fragments, 16S rDNA and 18S rDNA, widely used for the molecular identification of *Demodex* mites.

2. Materials and Methods

2.1. Sampling

Hair samples from Iberian and Italian wolves were obtained from northern Spain, including Cantabria (126 samples), Aragón (1), Castilla y León (1), and Catalunya (1), and from the Piemonte region of northern Italy (11) (Table 1). One hundred twenty-seven wolves (61 females and 66 males) from Cantabria and Castilla y León were morphologically and genetically identified as *Canis lupus signatus*, and 13 wolves (6 females and 7 males) from Piemonte, Catalunya, and Aragón were identified as *Canis lupus italicus* (Table 1). Between 2017 and 2025, we collected a total of 1,400 hair samples from 140 wolf carcasses, with 10 samples per carcass obtained during necropsy examinations (Table 1). Five sites were from the face: periocular, lips, nose, chin, and entrance to the external ear canal, and five sites were from the rest of the body: dorsum, lumbar, abdomen, forelimb, and hindlimb. Using gloved hands and surgical mosquito forceps, hair was plucked in the direction of growth to include the hair bulb (root) for DNA extraction. Hair samples were stored at -20ºC until DNA extraction.

Table 1. Sample collection

Host	Sample	Coll. Year	Sex	Collection Place	Positive areas	Nº sites	Nº sites (no DMX)	Nº sites (DMX)	DNA extraction	16S qPCR Detection	18S qPCR Detection	16S Tree name	GenBank (GB) number 18S	18S GB Identity	18S Tree name
Wolf A	CL01	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL02	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL03	2017	M	Ca-Spain	N/Li/C/A	4		4	Tris HCL	+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
Wolf A	CL04	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL05	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL06	2017	M	Ca-Spain	P/H	2		2	Tris HCL	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL07	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL08	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL09	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL10	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL11	2017	M	Ca-Spain	None				Tris HCL	-	-				

Wolf A	CL12	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL13	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL14	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL15	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL16	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL17	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL18	2017	F	Ca-Spain	P/N/L	3		3	Tris HCL	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL19	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL20	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL21	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL22	2017	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL23	2017	M	Ca-Spain	Li	1	1		Tris HCL	-	-		OR820194	100%	Phthiracarus_UABW
Wolf A	CL24	2017	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL25	2018	F	Ca-Spain	None				Tris HCL	-	-				

Wolf A	CL26	2018	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL27	2018	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL28	2018	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL29	2018	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL30	2018	F	Ca-Spain	Li	1	1		Tris HCL	-	-		GQ864286	100%	Adoristes_UABW
Wolf A	CL31	2018	M	Ca-Spain	Li	1		1	Tris HCL	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL32	2018	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL33	2018	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL34	2018	M	Ca-Spain	Li	1		1	Tris HCL	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL35	2018	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL36	2018	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL37	2018	M	Ca-Spain	Li	1		1	Tris HCL	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
Wolf A	CL38	2018	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL39	2018	F	Ca-Spain	P/Fo	2		2	Tris HCL	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW

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Wolf A	CL40	2018	F	Ca-Spain	Li	2		1	Tris HCL	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
	CL40				C			1		+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
Wolf A	CL41	2018	F	Ca-Spain	Li	1		1	Tris HCL	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
Wolf A	CL42	2018	F	Ca-Spain	P	1		1	Tris HCL	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL43	2018	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL44	2018	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL45	2018	M	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL46	2018	M	Ca-Spain	P	1		1	Tris HCL	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL47	2018	M	Ca-Spain	C	2	1	1	Tris HCL	+	-	D.canis_UABW1	Z74479	99,81%	Ixodes_UABW
	CL47				Fo		1			-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL48	2018	F	Ca-Spain	None				Tris HCL	-	-				
Wolf A	CL49	2018	M	Ca-Spain	P	2		1	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
	CL49				C		1		Perkins	-	-		HM070358	97,37%	Bdellodes_UABW
Wolf A	CL50	2018	F	Ca-Spain	N	1		1	Perkins	+	+	D.injai_UABW3	KU253789	100%	D.injai_UABW

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Wolf A	CL51	2018	F	CyL-Spain	None				Tris HCL	-	-				
Wolf A	CL52	2019	F	Ca-Spain	C	2		1	Perkins	+	+	D.injai_UABW3	KU253789	100%	D.injai_UABW
	CL52				P		1		Perkins	-	-		MK014972	100%	Camisia_UABW
Wolf A	CL53	2019	F	Ca-Spain	Li	2		1	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
	CL53				N		1		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL54	2019	F	Ca-Spain	L	1	1		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL55	2019	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL56	2019	F	Ca-Spain	P	1	1		Perkins	-	-		KR081607	100%	Damaeus_UABW
Wolf A	CL57	2019	M	Ca-Spain	P	1	1		Perkins	-	-		EU432193	99,34%	Lepidozetes_UABW
Wolf A	CL58	2019	M	Ca-Spain	D	1	1		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL59	2019	F	Ca-Spain	N	1	1		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL60	2019	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL61	2019	M	Ca-Spain	D	3		1	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
	CL61				H			1	Perkins	+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
	CL61				L		1		Perkins	-	-		KY922216	100%	Nothrus_UABW

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Wolf A	CL62	2019	M	Ca-Spain	Fo	1	1		Perkins	-	-		GQ864286	100%	Adoristes_UABW
Wolf A	CL63	2019	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL64	2019	M	Ca-Spain	E/D/L	3	3		Perkins	-	-		OM249668	99,54%	Penthaleus_UABW
Wolf A	CL65	2019	M	Ca-Spain	Li/N	2		2	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL66	2019	F	Ca-Spain	Li/Fo	2	2	2	Perkins	+	-	D.injai_UABW3	EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL67	2019	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL68	2019	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL69	2019	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL70	2019	F	Ca-Spain	A	1	1		Perkins	-	-		GQ864286	100%	Adoristes_UABW
Wolf A	CL71	2019	M	Ca-Spain	Li/N/C/L	4		4	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
Wolf A	CL72	2019	M	Ca-Spain	E/A	2	2	2	Perkins	+	-	D.folliculorum_UABW3	Z74479	99,81%	Ixodes_UABW
Wolf A	CL73	2019	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL74	2019	M	Ca-Spain	Fo	1		1	Perkins	+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
Wolf A	CL75	2019	F	Ca-Spain	E	1		1	Perkins	+	-	D.brevis_UABW1			

Wolf A	CL76	2019	M	Ca-Spain	A	2		1	Perkins	+	-	D.brevis_UABW1			
Wolf A	CL77	2020	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL78	2020	F	Ca-Spain	Li/C/D/Fo	4	4		Perkins	-	-		GQ864286	100%	Adoristes_UABW
Wolf A	CL79	2020	F	Ca-Spain	C/L	3		2	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
	CL79				Fo		1			-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL80	2020	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL81	2020	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL82	2020	F	Ca-Spain	N/E	2	2		Perkins	-	-		GQ864301	100%	Steganacarus_UABW
Wolf A	CL83	2020	F	Ca-Spain	P/N	2		2	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL84	2020	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL85	2020	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL86	2020	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL87	2020	F	Ca-Spain	Li/C	3		2	Perkins	+	+	D.injai_UABW3	KU253789	100%	D.injai_UABW
	CL87				P		1		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW

Wolf A	CL88	2020	F	Ca-Spain	P/Li	2		2	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
Wolf A	CL89	2020	M	Ca-Spain	P	1		1	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
Wolf A	CL90	2020	M	Ca-Spain	Li/N	2		2	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
Wolf A	CL91	2020	M	Ca-Spain	P/N/E/D/A/Fo/H	7	7		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL92	2020	F	Ca-Spain	P/N/C/E/D/A/Fo/H	8	8		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL93	2020	M	Ca-Spain	C/D/A	3	3		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL94	2020	F	Ca-Spain	P/Li/N/C/E/D/A/Fo/H	9		9	Perkins	+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
Wolf A	CL95	2020	M	Ca-Spain	C	8	1	1	Perkins	+	-	D.canis_UABW1	MK014972	100%	Camisia_UABW
	CL95				Fo		1	1	Perkins	+	-	D.brevis_UABW3	MK014972	100%	Camisia_UABW
	CL95				N/E/D/L/A/H		6			-	-		MK014972	100%	Camisia_UABW
Wolf A	CL96	2020	F	Ca-Spain	E	6	1	1	Perkins	+	-	D.folliculorum_UABW1	EU432204	99,81%	Ceratoppia_UABW
	CL96				P/Li/C/L/Fo		5			-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL97	2021	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL98	2021	M	Ca-Spain	E/D/L	3	3		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW

Wolf A	CL99	2021	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL100	2021	M	Ca-Spain	N/C	2		2	Perkins	+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
Wolf A	CL101	2021	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL102	2021	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL103	2022	M	Ca-Spain	P/Li/N/C/H	5		5	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL104	2023	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL105	2023	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL106	2023	F	Ca-Spain	C	3		1	Perkins	+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
	CL106				E/D		2	2		+	-	D.canis_UABW1	GQ864286	100%	Adoristes_UABW
Wolf A	CL107	2023	M	Ca-Spain	L/A/Fo	3		3	Perkins	+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
Wolf A	CL108	2023	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL109	2024	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL110	2024	M	Ca-Spain	Fo/H	2		2	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
Wolf A	CL111	2024	M	Ca-Spain	None				Perkins	-	-				

Wolf A	CL112	2025	F	Ca-Spain	P/Li	2		2	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
Wolf A	CL113	2025	M	Ca-Spain	None				Perkins	-	-				
Wolf A	CL114	2025	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL115	2025	F	Ca-Spain	C	1		1	Perkins	+	-	D.brevis_UABW2			
Wolf A	CL116	2025	M	Ca-Spain	Li	2		1	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
	CL116				A			1		+	+	D.canis_UABW1	KC010484	100%	D.canis_UABW
Wolf A	CL117	2025	F	Ca-Spain	C/D/L/A	4	4		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL118	2025	M	Ca-Spain	Li/N	2		2	Perkins	+	+	D.injai_UABW1	KU253789	100%	D.injai_UABW
Wolf A	CL119	2025	M	Ca-Spain	C/Fo/H	4		3	Perkins	+	+	D.canis_UABW2	KC010484	100%	D.canis_UABW
	CL119				L		1			-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL120	2025	M	Ca-Spain	E	2		1	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
	CL120				N		1		Perkins	-	-		OR820194	100%	Phthiracarus_UABW
Wolf A	CL121	2025	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL122	2025	M	Ca-Spain	None				Perkins	-	-				

Wolf A	CL123	2025	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL124	2025	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL125	2025	F	Ca-Spain	None				Perkins	-	-				
Wolf A	CL126	2025	F	Ca-Spain	N/C	3		2	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
	CL126				D		1		Perkins	-	-		EU432204	99,81%	Ceratoppia_UABW
Wolf A	CL127	2025	M	Ca-Spain	H	2		1	Perkins	+	+	D.injai_UABW2	KU253789	100%	D.injai_UABW
	CL127				Fo		1		Perkins	-	-		HM070361	100%	Anystis_UABW
Wolf B	CL128	2017	F	T-Italy	N/H	2	2		Tris HCL	-	-		LC817364	99,78%	Cymbaeremaeus_UABW
Wolf B	CL129	2017	M	Al-Italy	Li	1	1		Tris HCL	-	-		HM070361	100%	Anystis_UABW
Wolf B	CL130	2017	M	Cu-Italy	Li/N	3		2	Tris HCL	+	+	D.folliculorum_UABW2	KF745889	100%	D.folliculorum_UABW
	CL130				C			1	Tris HCL	+	-	D.brevis_UABW2			
Wolf B	CL131	2017	M	Cu-Italy	None				Tris HCL	-	-				
Wolf B	CL132	2017	F	T-Italy	None				Tris HCL	-	-				
Wolf B	CL133	2017	F	Cu-Italy	None				Tris HCL	-	-				
Wolf B	CL134	2017	F	T-Italy	N/C/E	3		3	Tris HCL	+	+	D.folliculorum_UABW1	KF745889	100%	D.folliculorum_UABW

Wolf B	CL135	2017	M	V-Italy	H	1		1	Tris HCL	+	-	D.folliculorum_UABW1			
Wolf B	CL136	2017	M	T-Italy	Li	1		1	Tris HCL	+	-	D.folliculorum_UABW1			
Wolf B	CL137	2018	F	Cu-Italy	E	2	1	1	Tris HCL	+	-	D.folliculorum_UABW2	EU432204	99,81%	Ceratoppia_UABW
	CL137				P		1		Tris HCL	-	-		AF022035	99,75%	Liodes_UABW
Wolf B	CL138	2018	F	Cu-Italy	None				Tris HCL	-	-				
Wolf B	CL139	2018	M	C-Spain	C	8	1	1	Tris HCL	+	-	D.folliculorum_UABW1	EU432190	99,41%	Chamobates_UABW
	CL139				E/D		2		Tris HCL	-	-		EU432204	99,81%	Ceratoppia_UABW
	CL139				P/Li/N/L/A		5		Tris HCL	-	-		EU432190	99,41%	Chamobates_UABW
Wolf B	CL140	2023	M	Ar-Spain	None				Perkins	-	-				

Wolf A (*Canis lupus signatus*); Wolf B (*Canis lupus italicus*); F (Female); M (Male); DMX (*Demodex*). P: Periocular; Li: Lips; N: Nose; C: Chin; E: Ear; D: Dorsum; A: Abdominal; L: Lumbar; Fo: Forelimb; H: Hindlimb. Ar: Aragón; Ca: Cantabria; CyL: Castilla y León; C: Catalunya; Cu: Cuneo; T: Torino; V: Vercelli.

2.2. DNA Extraction and Real-Time PCR (qPCR) Amplification

Over the years, DNA from hair bulbs was extracted using two different methods (Table 1): Tris HCl approach, following Ravera et al. 2011 [4]; and PerkinElmer automated extraction, Chemagic 360 Instrument (Baesweiler, Germany), following the manufacturer's protocol, including an incubation step at 56°C overnight and a final elution volume of 50 µL DNA. All extracted DNA was diluted 1:10 for qPCR amplification.

16S and 18S primers were used to amplify approximately 300-bp and 500-bp fragments of the mitochondrial *16S rRNA* gene and the nuclear *18S rRNA* gene, respectively [10]. All DNA samples were amplified by real-time qPCR and prepared under a laminar flow hood. Positive qPCR controls were obtained from known *Demodex* mite DNA [10]. Duplicates were amplified for each sample, and blank DNA extraction and negative qPCR controls were included to detect any qPCR exogenous DNA contamination. qPCR amplifications were performed in a QuantStudioTM 12K Flex Real-Time PCR System (Thermo Fisher Scientific). Amplicons were sequenced and purified when melting curves showed Tm = 75 ± 1 °C for the 16S *rRNA* gene and Tm = 83 ± 2 °C for the 18S *rRNA* gene, and amplification cycles had Cp < 37. Sequences were separated on an ABI PRISM 3730 automated sequencer (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.3. Genetic Variability and Phylogenetic Analysis

Genetic diversity in *Demodex* mites was analyzed using DNASP 5.10 [56]. For comparisons and phylogenetic analysis, we used mite sequences from wolves (those ending in UAB) and those available in GenBank. Sequences were analyzed using SEQSCAPE 2.1.1 software (Thermo Fisher Scientific) and were compared with the GenBank database (www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis for the mitochondrial *16S rRNA* gene was carried out using 44 sequences and 302 bp (gaps included). Phylogenetic analysis for the *18S rRNA* gene was carried out using 45 sequences and 491 bp (gaps included). The trees were rooted using outgroups from the class Pycnogonida, *Achelia hispida* (FJ862845) and *Ammothea* sp. (FJ862841) for the *16S rRNA* gene, and *Achelia echinata* (AF005438) and *Callipallene* sp. (AF005439) for the *18S rRNA* gene. MODELTEST 3.7 [57] was applied to select the best evolutionary model among 56 models of evolution by the Akaike information criterion. The Bayesian program MrBayes v3.2.7 [58] was implemented to generate the phylogenetic tree with 1,000,000 Markov Chain Monte Carlo iterations and a burn-in rate of 25%. The bootstrap values show the repeatability and accuracy of the three [59]. To display the phylogenies, we used the program FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

3.1. Mite Prevalence in Wolves

We screened 1,400 hair samples from 140 wild wolves for mite DNA targeting the *16S rRNA* and *18S rRNA* genes. qPCR samples were considered positive when the melting curves (Tm) were close to *Demodex* control values (Tm = 75 ºC ± 0.5 ºC, 16S rDNA; Tm = 81.5 ºC ± 0.5 ºC, 18S rDNA), and at least one gene was successfully sequenced. The prevalence of *Demodex* mites in the hairs of 140 wolves was 37% (52/140). 46% of the Italian wolves (6/13) were positive for *Demodex*, while 36% were positive among Iberian wolves (46/127) (Table 1). No bias toward females and males (χ^2 (1, N=140) = 1.02, $p > 0.05$) was observed among *Demodex*-positive wolves.

Sampling 10 sites, 101 out of 1,400 hair samples tested positive for *Demodex* by qPCR (Table 1). Twenty-three wolves (44%) were positive at one site, 19 wolves (36%) at two sites, 6 wolves (12%) at three sites, two wolves (4%) at four sites, and two wolves (2%) at five and nine sites, respectively. *Demodex* positivity was significantly higher in face samples (71%) compared to body samples (χ^2 (1, N=1,400) = 19.73, $p < 0.001$). The most likely site to detect *Demodex* mites was the lip area (20%), followed by the chin area (18%). The least common sites were the lumbar (4%) and the dorsal (3%) areas.

Regarding DNA extraction methods, the PerkinElmer automated extraction was significantly more efficient (χ^2 (1, N=1400) = 8.52, $p < 0.05$), with 71 positives out of 790 extractions (9%), compared to the This-HCL approach, which had 30 positives out of 610 extractions (5%). A significant disadvantage of the This-HCL method is that DNA is not purified, leading to fewer successful qPCRs due to increased inhibitor levels. However, its main advantages are speed and low cost.

3.2. Identification of *Demodex* spp.

Using the *18S rRNA* gene, 83 samples were positive for *Demodex* spp., 21 samples could not be identified due to different mite types in the same sample (double sequence), and 88 samples were positive for other acarid mites (Table 1). Among the positive *Demodex* samples, we identified *Demodex injai* (52), *Demodex canis* (26), and *Demodex folliculorum* (5), but not *Demodex brevis* (Tables 1 and 2). Using the *16S rRNA* gene, we identified 101 positive samples, including *D. injai* (54), *D. canis* (30), *D. folliculorum* (12), and *D. brevis* (5) (Tables 1 and 2).

Interestingly, the *Demodex* species commonly reported in dogs, such as *D. canis* and *D. injai*, were not detected in any of the Italian wolves examined. Instead, two species commonly associated with humans, *D. folliculorum* and *D. brevis*, were identified. Although differential sampling effort (13 Italian wolves versus 127 Iberian wolves) could potentially bias detection probabilities, the absence of *D. canis* and *D. injai* in Italian wolves was statistically significant (Fisher's exact test, p = 0.015), suggesting that this absence is unlikely to be attributable solely to sampling effort. One possible explanation would have been putative reduced contact rates between Italian wolves and domestic dogs compared with Iberian wolves, but this is inconsistent with a recent study that found evidence of extensive wolf-dog hybridization in peninsular Italy [60].

In contrast, all four *Demodex* species, those usually reported in both dogs and humans, were identified in Iberian wolves. *D. injai* showed the highest prevalence, present in more than half of the individuals (64%), followed by *D. canis* (24%), while *D. brevis* and *D. folliculorum* had lower rates (8% and 4%, respectively). Additionally, co-infestation with *D. canis* and *D. injai* and *D. canis* and *D. brevis* were observed in three and one Iberian wolves, respectively, and *D. brevis* and *D. folliculorum* in one Italian wolf (Tables 1 and 2).

3.3. 16S rDNA Sequence Variability and Phylogenetic Relationships

We identified 11 *Demodex* sequences (haplotypes) among 101 sequences of the mitochondrial *16S rRNA* gene from 52 wolves (Tables 1 and 2). The 11 *Demodex* haplotypes were submitted to GenBank because they had not been previously described in wolves and, in most cases, represented new records (Table 2).

Table 2. *Demodex* spp. detected by site using the *18S rRNA* and *16S rRNA* genes with the corresponding GenBank accession number, and *Demodex* variants identified, and the number of positive wolves using the *16S rRNA* gene.

***Demodex* sp.**	**N sites - 18S**	**GENBANK-18S**	**N Sites - 16S**	**Variants - 16S**	**GENBANK-16S**	**N Sites - 16S**	**N Iberian wolf - 16S**	**N Italian wolf - 16S**
D.brevis	0	-	5	D.brevis_UABW1	PX232547	2	2	
				D.brevis_UABW2	PX232548	2	1	1(5)
				D.brevis_UABW3	PX232549	1	1(3)	
D.folliculorum	5	PX421031	12	D.folliculorum_UABW1	PX232550	7	1	4
				D.folliculorum_UABW2	PX232551	3		2(5)
				D.folliculorum_UABW3	PX232552	2	1	

D.canis	26	PX421029	30	D.canis_UABW1	PX232553	27	11(1)(2)(3)(4)	
				D.canis_UABW2	PX232554	3	1	
D.injai	52	PX421030	54	D.injai_UABW1	PX232555	19	12(1)(4)	
				D.injai_UABW2	PX232556	29	16(2)	
				D.injai_UABW3	PX232557	6	4	
Total	**83**		**101**			**101**	**50**	**7**

(1-5) co-infested wolves.

They were identified as follows: 1) *Demodex injai* (three haplotypes): D.injai_UABW1 (N=19 sequences, identical to the sequence KT259449 from GenBank), D.injai_UABW2 (N=29 sequences), and D.injai_UABW3 (N=6 sequences); 2) *Demodex canis* (two haplotypes): D.canis_UABW1 (variant *cornei*) (N=27 sequences, identical to sequences JX390979 and MN161404 from GenBank), and D.canis_UABW2 (N=3 sequences, identical to several sequences from GenBank such as JF784000); 3) *Demodex folliculorum* (three haplotypes): D.folliculorum_UABW1 (N=7 sequences, identical to sequences HQ844221, FN424245, and FN424246 from GenBank), D.folliculorum_UABW2 (N=3 sequences), and D.folliculorum_UABW3 (N=2 sequences); and 4) *Demodex brevis* (three haplotypes): D.brevis_UABW1 (N=2 sequences), D.brevis_UABW2 (N=2 sequences), and D.brevis_UABW3 (N=1 sequence). Haplotypes from *D. injai* and *D. canis* were exclusive to the Iberian wolves, as well as haplotypes D.folliculorum_UABW3, D.brevis_UABW1, and D.brevis_UABW3 (Table 2). The exclusive haplotype found in Italian wolves was D.folliculorum_UABW2. Finally, haplotypes D.folliculorum_UABW1 and D.brevis_UABW2 were shared between wolf populations.

We conducted a phylogenetic analysis that included the 11 *Demodex* sequences, 31 sequences from the order Trombidiformes, and two outgroups obtained from GenBank (Fig 1). In total, we aligned 44 fragments of 302 bp each (gaps included). The Tamura-Nei substitution model, which assumes equal base frequencies and accounts for among-site rate heterogeneity using a gamma distribution (TrNef+G) was identified as the best-fit model for the *16S rRNA* gene.

The 16S-Bayesian tree (Figure 1) showed two major splits among the Trombidiformes order (bootstrap value 100%). All our samples belong to the Anystina cohort, which includes the family Demodecidae. However, the Demodecidae sequences did not cluster by host species. For instance, *D. brevis* (host: human) was sister taxa with *D. gatoi* (host: cat), and *D. folliculorum* clade (host: human) was sister to the *D. injai* clade (host: dog) with strong bootstrap support (100%). These results suggest that the evolutionary relationships among *Demodex* mites do not strictly follow host phylogeny, indicating possible host-switching events over evolutionary time.

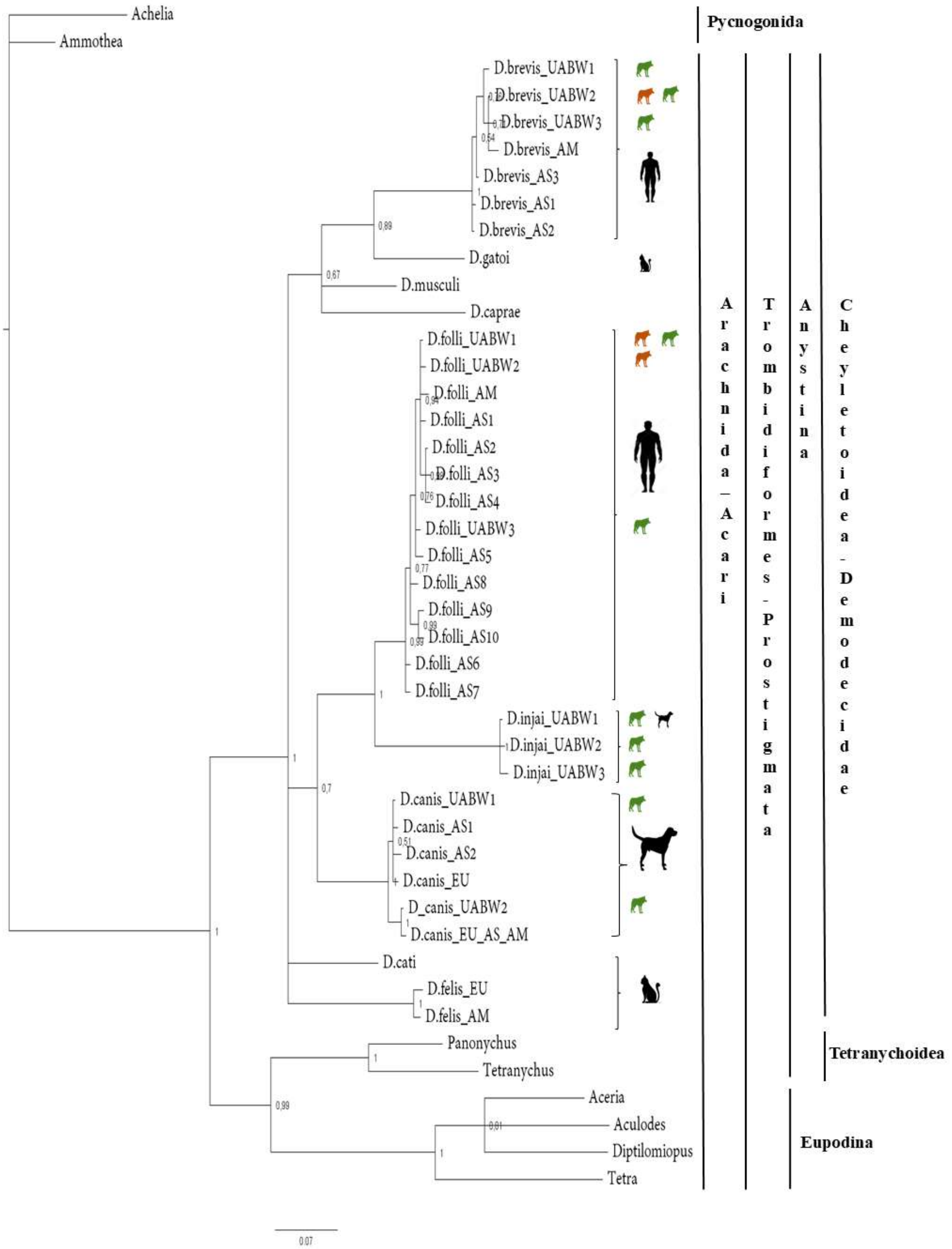

Figure 1. Phylogenetic analyses of *Demodex* spp. The tree was estimated using MrBayes based on aligned fragments of the *16S rRNA* gene. Branch support is based on 10,000 bootstrap replications. The scale at the bottom represents genetic distances, expressed as the number of nucleotide substitutions per site.

3.4. 18S rDNA Sequence Variability and Phylogenetic Relationship

We identified three *Demodex* sequences (haplotypes) among 83 sequences of the nuclear *18S rRNA* gene from 40 wolves (Tables 1 and 2). The three *Demodex* haplotypes were submitted to GenBank because they had not been previously described in wolves (Table 2). They were identified as *Demodex injai*, *Demodex canis* and *Demodex folliculorum*, and they have been described previously in dogs and humans (Table 1).

In addition to *Demodex*-positive samples, we sequenced 49 samples that amplified at Cp < 37 and melting curves Tm = 82 ºC + 3 ºC (Table 1). We amplified DNA from 11 mites belonging to the order Oribatida, 3 to the order Prostigmata, and one to the order Ixodida, the only hematophagous mite detected in an Iberian wolf (Table 1). The remaining mites were most likely soil-dwelling and not host-associated. Table 1 shows the sequences of the 15 mites, along with their percentage identity relative to the reference sequence in GenBank. The genus names used in the phylogenetic tree should be considered tentative, as the order-level classification is reliable, but species-level identification is not possible with the available data. These findings indicate that the 18S rDNA fragment is not an optimal marker for detecting *Demodex* in wolves.

The 18S-Bayesian tree (Figure 2) showed a clear split (bootstrap value 100%) between the Parasitiformes (Ixodida) and the Sarcoptiformes (Oribatida) and the Trombidiformes (Prostigmata) Orders. However, Oribatida did not form a distinct cluster, whereas Prostigmata split into several clusters, including the Eupodina and Anystina cohorts. The Demodecidae family constitutes a monophyletic group of the Anystina cohort that includes four large clusters: 1) the *D. folliculorum* cluster (host: human), 2) the *D. canis* cluster (host: dog), 3) the *D. gatoi*, *D. cati,* and an unnamed *Demodex* species in a cat (here referred to as *D. felis*) cluster (host: cat), and 4) the *D. brevis* cluster (host: human). *D. injai* appears to be more closely related to *D. folliculorum* than to *D. canis;* however, the bootstrap value (<70%) should be considered with caution.

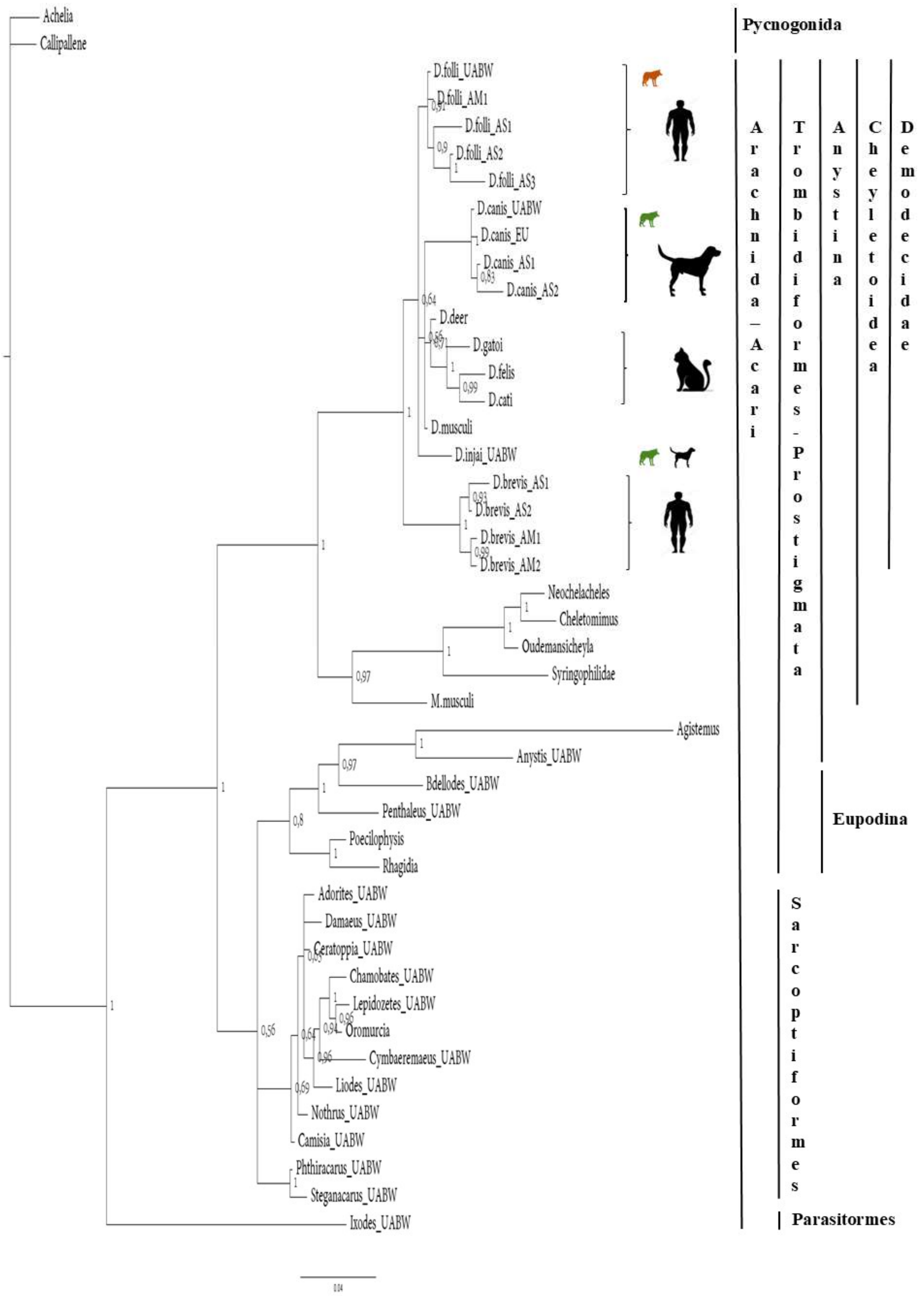

Figure 2. Phylogenetic analyses of *Demodex* spp. The tree was estimated using MrBayes based on aligned fragments of the *18S rRNA* gene. Branch support is based on 10,000 bootstrap replications. The scale at the bottom represents genetic distances, expressed as the number of nucleotide substitutions per site.

4. Discussion

4.1. Detection, Prevalence, and Identification of *Demodex* spp.

Demodex mites were detected in over one-third of the wolves, with a slightly higher prevalence in Italian than in Iberian wolves and no sex bias, indicating widespread infestation in both populations. It is important to emphasize that the wolves in this study were wild. In a previous study, in which we examined five body regions from 14 Mexican (*Canis lupus baileyi*) and 8 Alaskan (*Canis lupus occidentalis*) captive wolves, we were unable to detect any positive cases with confidence [10]. The fact that these captive animals were vaccinated annually, fed regularly, and kept in sheltered conditions possibly reduced mite proliferation and, consequently, the likelihood of detection, despite our certainty that all wolves harbor *Demodex* mites in their pilosebaceous complexes. Notably, to detect 37% of wolves positive for *Demodex*, each wolf had to be sampled at 10 different skin sites, yielding a total of 1,400 samples, of which only 7% tested positive. Therefore, it is more effective to examine fewer wolves across multiple sites than to explore a larger number of wolves at a single site [1,10,15]. Based on our results, the number of sampling sites for detecting *Demodex* mites can be limited to the face, with the most suitable sites being the lips and chin. The dorsal and lumbar regions can be excluded. Reducing the number of sites decreases laboratory workload and overall costs. The resulting time and resources are recommended for use in a DNA extraction method incorporating a purification step, despite its higher cost relative to non-purified DNA extraction. Regarding the genetic markers, our results suggest that the *16S rRNA* gene is more suitable for *Demodex* detection and phylogenetic inference than the *18S rRNA* gene. Amplification of the 18S marker may lead to the unintended detection of non-target mites, such as soil mites, or, in some cases, to the failure to detect *Demodex* mites. The mitochondrial 16S marker, instead, showed greater sequence variability and, consequently, higher resolution at short evolutionary distances than the nuclear 18S marker. However, despite this increased variability, bootstrap support for the nodes separating the major *Demodex* lineages remained low, generally failing to reach the 0.9–0.95 threshold, except for the *folliculorum–injai* clade. A similar lack of strong support was observed in the 18S-based phylogeny. These results indicate that some uncertainty persists in the inferred relationships among *Demodex* species, highlighting the need for additional loci and increased sampling to robustly resolve species boundaries and evolutionary relationships within the genus.

4.2. Co-Infestations and Host Specificity Concerns

To date, no peer-reviewed studies have explicitly documented co-infestations of *D. canis* and *D. injai*, and *D. canis* and *D. brevis*, within canids. Most reports describe these species in isolation, as dogs exhibit clinical signs of demodicosis [1,61,62]. Similarly, studies conducted in healthy dogs have not reported co-infestations [15,23,24]. In our study, no known diseases were present in either population at the time, which supports previous findings that *Demodex* mites can be carried asymptomatically across various mammalian hosts [10,15,63]. The absence of confirmed co-occurrence in dogs may indicate underdiagnosis due to limitations in sampling strategies, as co-infestations of *D. canis* with *D. folliculorum* and *D. brevis* have been documented in two healthy cats [8]. Co-infestation of *D. brevis* and *D. folliculorum* has also been documented in humans, particularly in relation to dermatological and ophthalmological conditions, such as chronic blepharitis and rosacea [64–66]. These two species usually inhabit different ecological niches: *D. folliculorum* in hair follicles and *D. brevis* in sebaceous or meibomian glands, but they can coexist in the same individual [66], and their combined presence has been suggested as a potential factor in the development of these diseases [67,68].

The observation of interspecific co-infestations in some wolves, along with findings that the Iberian wolf harbours *Demodex* species found in dogs and humans, suggests substantial ecological plasticity and raises important questions about the strict host specificity paradigm. Previous studies have raised similar concerns by reporting the presence of *D. canis* not only in dogs and wolves, but also in a variety of hosts, including bats, ferrets, cats, and mice, and, exceptionally, in a single bear and a single human [8,10,13,34,69]. These findings support a revision of the host-parasite model for

Demodex, proposing that at least some species, especially those infecting carnivores, may function as opportunistic generalists rather than obligate specialists. Molecular studies across a broader range of wild and domestic hosts are needed to further assess the extent of host specificity versus host plasticity in this group.

4.3. Inference on the Process of Dog Domestication

The identification of *D. canis*, *D. injai*, *D. folliculorum*, and *D. brevis* in wild wolves is a remarkable finding that challenges the traditional view of strict host specificity in *Demodex* mites. While *D. canis* and *D. injai* are typically associated with domestic dogs, and *D. folliculorum* and *D. brevis* with humans, their concurrent presence in wolves raises several questions regarding their evolutionary history and host associations. The detection of *D. canis* and *D. injai* in Iberian wolves is fully expected, whereas their absence from the skin of Italian wolves is unexpected. Although these mites should have been detected statistically, further analysis of samples would be necessary to confirm their presence in this wolf population. And even more surprising is the detection of human-associated *Demodex* in wolves. Two possible explanations can be proposed for this unexpected detection: 1, wolves served as a reservoir and potential source of transmission to humans, occurring through early interactions between canids and hominids during the process of dog domestication [2], and 2, a more plausible scenario involving ecological contact and host-switching events, given the recent isolation history of these two wolf populations. The Iberian and Italian wolf populations have been geographically isolated since the early 20th century, when wolves became extinct in France [55]. Since then, the population sizes of Spanish and Italian wolves have also declined dramatically, with minima reached in both countries during the 70s of the 20th century [54,55,70,71]. The presence of human-associated *Demodex* species in wolves may result from cross-species transmission, potentially facilitated by overlapping habitats, direct contact (e.g., in captivity or rehabilitation centers), or indirect contact through domestic dogs. However, to date, no reports have been published of dogs harboring human-associated *Demodex* on their skin. Future studies employing longer or more variable genetic markers alongside broader host and geographic sampling will be crucial to disentangle the evolutionary relationships of *Demodex* mites, clarify potential host-switching events, and enhance our understanding of their ecology and evolution in both wild and domestic hosts.

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Institutional Review Board Statement: In Cantabria, wolves were classified as a hunting species under Annex I of the Cantabrian Law, 12/2006. From 2017 to 2020, wolves were removed under a species-control program implemented by “Subdirección General del Medio Natural” of the Government of Cantabria, in accordance with Laws 2/2017, 5/2018, and MED/5/2019, under expedient numbers CVE-2017-1827, CVE-2018-17138, and CVE-2019-2950, to reduce wolf attacks on livestock in conflict areas. From 2021 to 2025, wolves in the Iberian Peninsula were protected, and hunting was banned. Individuals necropsied from 2021 onwards died accidentally. Natural Environment agents transported the carcasses to the Wildlife Rescue Center of Cantabria for necropsies. The two wolves from Castilla y León and Catalunya died after being hit by a car. Both carcasses were transported by Natural Environment agents to the UAB for necropsy. Individuals from Italy (where wolf hunting is banned) died accidentally. Technicians from the European project Life12 NAT/IT/000807 WolfAlps moved carcasses to the Department of Veterinary Science (Università degli Studi di Torino) for necropsies. All procedures involving animal carcasses complied with national and regional regulations and did not involve the deliberate killing of animals for research purposes.

Informed Consent Statement: Not applicable.

Data Availability Statement: All relevant data are within the paper. All new sequences are available from the GenBank database (accession numbers: PX232547, PX232548, PX232549, PX232550, PX232551, PX232552, PX232553, PX232554, PX232555, PX232556, PX232557, PX421029, PX421030, PX421031).

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Abbreviations

The following abbreviations are used in this manuscript:

qPCR	Quantitative Polymerase Chain Reaction
Tm	Melting curve
Cp	Amplification cycle
SNP	Single Nucleotide Polymorphism
UAB	Universitat Autònoma de Barcelona
IUCN	International Union for Conservation of Nature
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora

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