



Unexpected *Echinococcus multilocularis* infections in shepherd dogs and wolves in south-western Italian Alps: A new endemic area?



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ABSTRACT

The European distribution of *Echinococcus multilocularis* has been reported to be expanding east and north, whereas its southern limits are deemed stable. During a study on *Echinococcus granulosus* s.l. infections in wolves and dogs in the Italian Maritime Alps, we unexpectedly detected the presence of *E. multilocularis* eggs in four fecal samples from at least two shepherd dogs, and in five wolf fecal samples. This finding, in an area about 130 km south of the southernmost *E. multilocularis* report in the Alps, may suggest a rapid expansion southward. While infections in foxes are currently being investigated, these data seem to indicate the potential for a new *E. multilocularis* endemic area. If this will be confirmed, the implementation of surveillance programs in wild and domestic canids and preventative measures will become a priority.

1. Introduction

Echinococcus multilocularis, a tapeworm with an indirect life cycle involving mammalian predators as definitive hosts (primarily wild or domestic canids; DH hereafter) and mammalian prey species as intermediate hosts (primarily small rodents; IH hereafter; reviewed in (Romig et al., 2017)), is the etiological agent of alveolar echinococcosis (AE hereafter), the third most relevant human foodborne parasitic disease worldwide (FAO/WHO, 2014). Once mature, the adult worms, attached to the intestinal tract of the DHs, release in the host intestinal lumen their last proglottids with hundreds of embryonated eggs that will be later excreted in the environment within the host feces. Once ingested by a competent IH, or by people (as dead-end host), the eggs hatch and release larvae (oncospheres) in the intestinal tract of the host that pass through the intestinal wall into the blood vessels, and from there to the target organs (almost exclusively the liver). There the oncospheres will develop into metacestodes that will replicate asexually producing thousands of infectious larvae (protoscoleces). When the

infectious IH is preyed upon by a competent DH, the protoscoleces evaginate, attach to the wall of the small intestine and develop into adults. The asexual multiplication phase of the metacestode creates very severe clinical conditions, due to an infiltrating tumor-like process that damage the tissues and will eventually invade other organs through a metastasis-like process (Kern et al., 2017). Human AE is estimated to affect more than 18,000 new patients worldwide every year (Torgerson et al., 2010), with about 150–200/year expected in central Europe alone, and an incremental trend in the last decades (Vuitton et al., 2015). Human AE is deadly if untreated, and still highly pathogenic with a fatality of 16% despite recent advances in treatment (Kern et al., 2017). In Europe the parasite is distributed mostly in its central and eastern part, extending into northern France, southern Scandinavia (Denmark, southern Sweden) and parts of the Balkan peninsula (Combes et al., 2012; Deplazes et al., 2017; EFSA, 2015; Oksanen et al., 2016; Umhang et al., 2016).

At present, the parasite is deemed to be expanding north and east, but its southern limits, at least in the Alpine region, are described as

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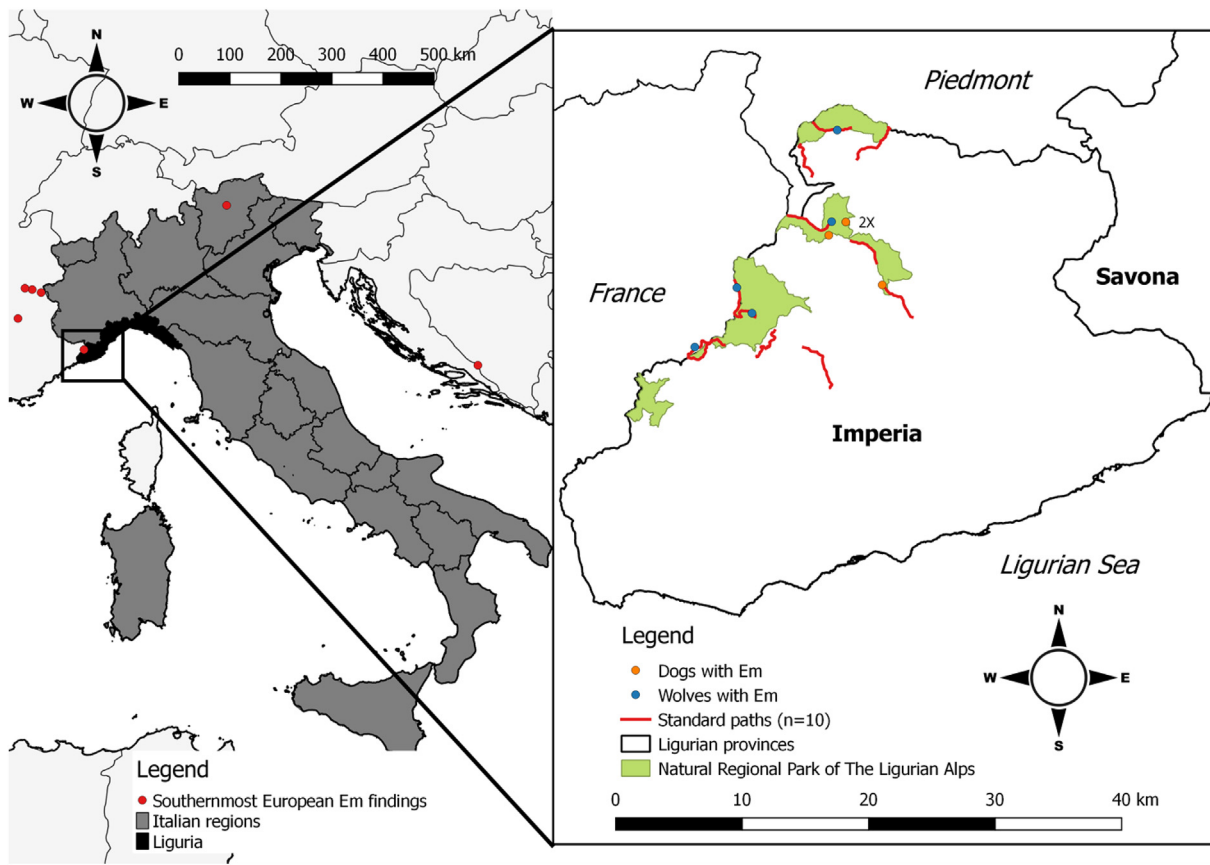


Fig. 1. Locations of wolf (blue dots) and dog (orange dots) fecal samples positive to *Echinococcus multilocularis* collected during a survey on *Echinococcus* spp. carried out from June to November 2017 in a mountainous area in the Alps of the Imperia Province, Italy. In the map are also reported the southernmost reports of *Echinococcus multilocularis* (*E. multilocularis*) to date in Europe (France, Drs. Boué and Umhang, pers. communication; North-Eastern Italian Alps, Croatia, as in (Beck et al., 2018)). Two dog fecal samples were collected from the same pasture and are represented by a single dot (noted as 2 ×). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stable (Conraths and Deplazes, 2015) and some authors suggested that the limited distribution of *Microtus arvalis* in southern slopes of the Alps may play a role in limiting *E. multilocularis* distribution in Switzerland (Guerra et al., 2014). Recent findings of *E. multilocularis* infections in foxes from Southern France in the Hautes Alpes Department (05) seem to indicate a possible expansion southward (Combes et al., 2012; Umhang et al., 2016).

In Italy, the only reports of *E. multilocularis* infections have been in the eastern Alps in the Trentino-Alto Adige Region (EFSA, 2015) where multi-locus microsatellite analysis suggested an autochthonous focus of the parasite (Casulli et al., 2009), and where a recent meta-analysis based on 26 papers indicated a prevalence in foxes between 0.5 and 2.9 (Oksanen et al., 2016).

2. Material and methods

From June to November 2017, we conducted a fecal survey of taeniid cestodes in wild and domestic canids in a protected area (Parco Regionale delle Alpi Liguri) of the Southern Italian Alps in the Imperia Province (Liguria region), about 25 km from the Mediterranean coast in Italy (Fig. 1).

The area is located in north-western Italy and connects the Apennines with the Maritime Alps (44°10'N 8°05'E; 6041 ha). The elevation ranges from 0 to 2200 m a.s.l. Chestnut and beech woods are the predominant forests. Meso-fauna includes wolf *Canis lupus*, fox *Vulpes vulpes*, and dogs *Canis lupus familiaris* as predators, and roe deer *Capreolus capreolus*, chamois *Rupicapra rupicapra*, and the wild boar *Sus scrofa*. Livestock amounts to more than 3200 cattle, 1200 sheep and

goats, and ca 50 horses. Potential IH species for *E. multilocularis* includes *Arvicola scherman*, *Chionomys nivalis*, *Microtus multiplex*, *Microtus savii*, *Myodes glareolus*, *Apodemus alpicola*, *Apodemus flavicollis*, *Apodemus sylvaticus*, *Mus musculus* along with *Rattus norvegicus* and *Rattus rattus*.

We identified 10 standard pathways (4.3 ± 0.27 km) that were walked on a monthly basis to locate and collect feces of wolves and domestic dogs, particularly those owned by local shepherds.

In order to correctly discriminate wolf feces to those of dogs, molecular host identification was performed purifying the genomic DNA with the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) and analyzing the 281 bp sequence of the mtDNA control region (Sindičić et al., 2011).

Samples were stored at -80°C for at least 3 days to deactivate the eggs (Veit et al., 1995), then kept at -20°C until subsequent analyses.

The samples were first screened for taeniid eggs using ZnCl_2 flotation followed by sieving (Mathis et al., 1996). Single eggs were separated and molecularly characterized using partial sequences of the *nad1* mtDNA gene as marker (Hüttner et al., 2008) (Table 1). Egg samples which yielded no positive result after *nad1* PCR were further analyzed targeting sequences of the *cob* gene (Hüttner et al., 2008) (Table 1). Readable sequences of partial *nad1* and *cob* were aligned using MEGA7 (Kumar et al., 2016) and compared to GenBank retrieved homologous sequences of *Echinococcus multilocularis*, *E. granulosus*, *E. ortleppi*, *E. canadensis*, *Taenia hydatigena*, *T. krabbei* and *T. ovis*, according to availability.

Table 1

Primer pairs used for PCR to molecularly characterize *Echinococcus multilocularis* in canid fecal samples from the Imperia area in Liguria, Italy, in 2017.

Target genes	Primers for the first PCR (5'-3') ^a	Primers for the second PCR (5'-3')
<i>nad1</i> (mtDNA) ^b	F: TGTTTTGAGATCAGTTCGGTGTG R: CATAATCAAACGGAGTACGATTAG	F: CAGTTCGGTGTGCTTTTGGGTCTG R: GAGTACGATTAGTCTCACACAGCA
<i>cob</i> (mtDNA) ^b	F: TTATGCTATACTTCGGTGTATTA R: ATAAGGATACTCCGGATGACAAC	F: TCGGTGTATTAATTCGAAGATTG R: GATGACAACCACCAATAAGTC

^a F, forward primer; R, reverse primer.

^b Primers designed by (Hüttner et al., 2008).

3. Results and discussion

We collected a total of 183 fecal samples. Definitive host species identification by sequencing part of the mtDNA control region confirmed field observations for all the *E. multilocularis*-positive dog fecal samples, while one wolf fecal sample (IM WLF 79) out of six *E. multilocularis*-positives turned out to be of a dog so we ended up with 151 feces of wolves and 32 of dogs. We then could screen 152/183 feces (wolf, 120; dog, 32; the rest were discarded from the analyses as they were either too small to allow for both diet analysis and parasitology, or too dry and old) and detected Taeniidae eggs in 11.66% of wolf feces (14/120) and 15.62% of dog feces (5/32).

We isolated 10 to 20 single taeniid eggs from 14 wolf fecal samples and from 5 dog fecal samples. Four out of five dog fecal samples

contained *E. multilocularis* eggs along with other Taeniidae infections (*Taenia hydatigena*, *T. krabbei*, *T. ovis*; Table 2), whereas only 5 out of the 14 wolf fecal samples contained *E. multilocularis* eggs, and showed coinfection with *Taenia* spp. and *E. orteppi* in two samples (see sequences alignments for positive specimens of *E. multilocularis* at partial *cob* Fig. 2a and b; and for positive specimens of *E. orteppi* at partial *nad1*, Fig. 2c).

Importantly, some fecal samples of both wolf and dog were from the same individuals, but we are sure that the dog samples came from at least two different individuals belonging to two different shepherds, and not allowed to roam at night. No travel history that may justify allochthonous infections was reported for these shepherd dogs.

Although we cannot exclude that some of the wolf fecal samples were from the same animal, previous studies have indicated the

Table 2

Molecular analysis results conducted on eggs harvested from fecal samples of wolves (WLF; upper table) and dogs (DOG; lower table) collected from June to November 2017 in a mountainous area in the Alps of the Imperia Province, Italy. In table are reported the total number of eggs harvested in each sample, the number of eggs positive (+ve) to *nad1* and *cob* and the corresponding sequencing results.

ID	N Taeniid Eggs extracted	<i>nad1</i>		<i>cob</i>	
		N +ve eggs	sequencing results	N +ve eggs	sequencing results
IM WLF 42	7	0	NA	2	42.4 <i>Echinococcus multilocularis</i> 42.7 <i>Taenia hydatigena</i>
IM WLF 88	10	6	L 88.2 <i>Echinococcus orteppi</i> L 88.4 <i>Echinococcus multilocularis</i> L 88.5 <i>Echinococcus orteppi</i> L 88.7 <i>Taenia krabbei</i> L 88.8 <i>Echinococcus orteppi</i> L 88.10 <i>Taenia krabbei</i>	0	NA
IM WLF 94	10	6	94.1 <i>Taenia hydatigena</i> 94.2 <i>Taenia hydatigena</i> 94.3 <i>Taenia hydatigena</i> 94.4 <i>Taenia ovis</i> 94.5 <i>Taenia hydatigena</i> 94.8 <i>Taenia hydatigena</i>	1	94.10 <i>Echinococcus multilocularis</i>
IM WLF 107	10	0	NA	3	107.1 <i>Taenia krabbei</i> 107.2 <i>Taenia hydatigena</i> 107.4 <i>Echinococcus multilocularis</i>
IM WLF 143	10	8	143.1 <i>Taenia hydatigena</i> 143.2 <i>Taenia hydatigena</i> 143.3 <i>Echinococcus multilocularis</i> 143.4 <i>Taenia hydatigena</i> 143.5 <i>Taenia hydatigena</i> 143.7 <i>Echinococcus orteppi</i> 143.8 <i>Taenia hydatigena</i> 143.9 <i>Echinococcus orteppi</i>	0	NA
IM WLF 79 (DOG ^a)	7	3	79.1 <i>Taenia ovis</i> 79.2 <i>Taenia ovis</i> 79.6 <i>Taenia ovis</i>	4	79.3 <i>Taenia ovis</i> 79.4 <i>Echinococcus multilocularis</i> 79.5 <i>Echinococcus multilocularis</i> 79.7 <i>Taenia ovis</i>
DOG 11	9	3	C 11.2 <i>Taenia hydatigena</i> C 11.7 <i>Echinococcus multilocularis</i> C 11.8 <i>Taenia hydatigena</i>	0	NA
DOG 14	10	4	14.6 <i>Taenia hydatigena</i> 14.7 <i>Echinococcus multilocularis</i> 14.9 <i>Taenia hydatigena</i> 14.10 <i>Taenia hydatigena</i>	1	14.8 <i>Echinococcus multilocularis</i>
DOG 30	10	2	30.3 <i>Taenia hydatigena</i> 30.9 <i>Echinococcus multilocularis</i>	0	NA

^a Initially classified as wolf.

A	CANE14_8_cob_Em	GGG GGT TGT TGT TGA TTT TAT CTT TTA TTT TTT TCT TAT GAG TAC CAA	[48]
	IMCL79_5_Em	...	[48]
	IMCL94_10_Em	...	[48]
	MF370870_Em	...	[48]
	MF370869_Em	...	[48]
	AB461400_Em	...	[48]
	AB374426_Em	...	[48]
	AB510026_Em	.T.C	[48]
	AB461402_Em	.T.C	[48]
	CANE14_8_cob_Em	CTG AGG GTG GCA CTA GTG TTT ATA GAG TTT GGC GTC AGG TTA ATT TTT	[96]
	IMCL79_5_EmC.	[96]
	IMCL94_10_Em	[96]
	MF370870_Em	[96]
	MF370869_Em	[96]
	AB461400_Em	[96]
	AB374426_Em	[96]
	AB510026_Em	[96]
	AB461402_Em	[96]
	CANE14_8_cob_Em	GGT TAG TTG TTA GTT TGT TTT TGT CAT T	[124]
	IMCL79_5_Em	[124]
	IMCL94_10_Em	[124]
	MF370870_Em	[124]
	MF370869_EmG	[124]
	AB461400_EmA.	[124]
	AB374426_EmA.	[124]
	AB510026_EmA.	[124]
	AB461402_EmA.	[124]

Fig. 2. Multiple alignment of partial mitochondrial *cob* (124bp) from three specimens identified as *Echinococcus multilocularis* analyzed in the present paper (the first three input sequences) with: (a) six *E. multilocularis* sequences retrieved from GenBank after comparison by Local Alignment Search Tool BLAST; (b) five sequences referred to *Echinococcus granulosus* (Eg, EgG1), *E. ortleppi* (EgG5), *E. canadensis* (EgG6-7) and *Taenia hydatigena* (*Thy*) retrieved from GenBank. (c) Multiple alignment of partial mitochondrial *nad1* (139bp) from three specimens identified as *Echinococcus ortleppi* analyzed in the present paper (the first three input sequences) with sequences retrieved from GenBank belonging to other representatives of *E. ortleppi*, *E. granulosus*, *E. canadensis*, *E. multilocularis*, *T. krabbei*, *T. ovis* and *T. hydatigena*. Dots indicate identity with nucleotide of the first sequences listed.

presence of at least two or more distinct wolf packs in the areas we investigated (Imbert et al., 2016; Marucco and Avanzinelli, 2017), so we believe that the positive wolf fecal samples are from more than one individual. Individual wolf genotyping from our fecal samples was not conducted because of lack of sufficient fecal matter after the repeated

molecular testing.

Whereas the *Taenia* spp. infections were known to be common in these areas (Gori et al., 2015), the detection of *E. ortleppi* (G5) is unexpected. This is the first report of G5 in Italy in wolves, and in general in DHs in this country. The cycle of this parasite is mainly cattle–dog,

B	CANE14_8_cob_Em	GGG GGT TGT TGT TGA TTT TAT CTT TTA TTT TTT TCT TAT GAG TAC CAA	[48]
	IMCL79_5_Em	...	[48]
	IMCL94_10_Em	...	[48]
	AB786664_Eg	.T.C ..CA .G.TC .G. ..AG.	[48]
	AF297617_EgG1	.T.C ..CA .G.TC .G. ..AG.	[48]
	NC_011122_EgG5	.C.A.G .G.CGG. .G. ..AT.	[48]
	MH300954_EgG6	.T.G .G.CGA. .G.T.	[48]
	MH301022_EgG7	.T.G .G.CGA. .G.T.	[48]
	GQ228819_ThyA. .AG .A.AG CT.T .A.A. .G. ..A .T. .T.	[48]
	CANE14_8_cob_Em	CTG AGG GTG GCA CTA GTG TTT ATA GAG TTT GGC GTC AGG TTA ATT TTT	[96]
	IMCL79_5_EmC.	[96]
	IMCL94_10_Em	...	[96]
	AB786664_Eg	.A. .A.GT .G.G. .G.	[96]
	AF297617_EgG1	.A. .A.GT .G.G. .G.	[96]
	NC_011122_EgG5A. .C. .GTA.AA.A .A.	[96]
	MH300954_EgG6T.GTA.A .A.	[96]
	MH301022_EgG7T.GTA.A .A.	[96]
	GQ228819_Thy	..A GTA A.. .TTA. .C. AT. .G.AT .G. .A. ...	[96]
	CANE14_8_cob_Em	GGT TAG TTG TTA GTT TGT TTT TGT CAT T	[124]
	IMCL79_5_Em	...	[124]
	IMCL94_10_Em	...	[124]
	AB786664_Eg	.A. ..AG.A.G. .	[124]
	AF297617_EgG1	.A. ..AG.A.G. .	[124]
	NC_011122_EgG5AG.A. .C.GC .	[124]
	MH300954_EgG6GAA.A.G. .	[124]
	MH301022_EgG7GAA.A.G. .	[124]
	GQ228819_Thy	.A. .GATA.T. .T. .	[124]

Fig. 2. (continued)

but this species has also been identified in other IHs such as buffaloes, pigs, sheep and humans as dead-end hosts (Dinkel et al., 2004; Romig et al., 2017). *Echinococcus ortleppi* was reported for the first time in Italy by Casulli and colleagues (Rinaldi et al., 2008) in one bovid, and infections without fertile cysts of the cattle strain were reported in Campania (Rinaldi et al., 2008). Definitely, considering the potential

for zoonotic infections, further investigation in dogs and wolves is to be implemented in this area to understand which wild and domestic IH species are involved in the maintenance of the parasite in the environment.

Finally, but more importantly, the finding of *E. multilocularis* in both dogs and wolves was not only unprecedented, but also surprising and

C IMCL143_7_nad1_G5	TTA GGT TTG AGG CTT GTT TTA TGT GTG TGG TTA TTT TTT GTG CTT TGT	[48]
IMCL113_7nad1_G5C. ...	[48]
ALPECL37_2nad1_G5	[48]
MG271922_Eortleppi	[48]
LC167081_Eortleppi T. ...	[48]
KT363810_Eortleppi	[48]
AJ237636_Eortleppi	[48]
AB979274_Eortleppi	[48]
EU704122_EmA.A.T ...	[48]
AB668376_EmA.A.T ...	[48]
KT988120_EgranulosusC.A.	[48]
KJ663949_EgranulosusA.C.A.	[48]
KX010880_EcanadensisC.A.	[48]
EU544632_Tkrabbei	.A.A .T.A. ... C.. .G. .A.	[48]
MH287111_Tovis	.A.A .T. .C. .A. ... C.. .A. ...	[48]
JN831284_Thydatigena	.A.T. CGT .G. ..A	[48]
AJ277409_Thydatigena	.A.T. CGT .G. ..A	[48]
AJ277408_Thydatigena	.A.T. CAT .G. ..A	[48]
IMCL143_7_nad1_G5	GTT GTT GTA GGT ATA ATT TAA TTG ATT TTT ATC ATA GTT ACT GGT GGA	[96]
IMCL113_7nad1_G5	[96]
ALPECL37_2nad1_G5C	[96]
MG271922_Eortleppi	[96]
LC167081_Eortleppi	[96]
KT363810_Eortleppi	[96]
AJ237636_Eortleppi	[96]
AB979274_Eortleppi	.C.	[96]
EU704122_Em	... AC.G.T GT. .A. .A.	[96]
AB668376_Em	... AC.G.T GT. .A. .A.	[96]
KT988120_EgranulosusGT	[96]
KJ663949_EgranulosusGT.	[96]
KX010880_EcanadensisGT	[96]
EU544632_Tkrabbei	.C. A.. A.. .T. ... G.. .GG .G.C. T.T TG. .CA GT. ... TAT	[96]

Fig. 2. (continued)

D

MH287111_Tovis	... A..T.GG .G. T.T T.. .CG GT. ... TAT [96]
JN831284_Thydatigena	.G. A.. T.. .T.GG A.. T.T TG. ..G .T. ... TCT [96]
AJ277409_Thydatigena	.G. A.. T.. .T.GG A.. T.T TA. ..G .T. ... TTT [96]
AJ277408_Thydatigena	.G. A.. T.. .T.GG A.. T.T TA. ..G .T. ... TTT [96]
IMCL143_7_nad1_G5	GTT GGT TAT TAT TAC CAT TAA TTT ATG GGT TAT TCT TGG TGT G [139]
IMCL113_7nad1_G5T. [139]
ALPECL37_2nad1_G5T. [139]
MG271922_EortleppiT. [139]
LC167081_EortleppiT. [139]
KT363810_EortleppiT. C.. C.. [139]
AJ237636_EortleppiT.T. [139]
AB979274_EortleppiT. [139]
EU704122_Em	.C. T.. .G. .G. .T.G.A. .G. .T. [139]
AB668376_Em	.C. T.. .G. .G. .T.G.A. .G. .T. [139]
KT988120_EgranulosusA.C. [139]
KJ663949_EgranulosusA.C. [139]
KX010880_EcanadensisC. [139]
EU544632_Tkrabbei	CGG TTA .GG .T. .T. .T. GTT .G. TGA TT. ... ATG .AA .A. [139]
MH287111_Tovis	CAA TTG .GA .T. .T. .G. GTG ..G TA. TT. ... AT. .A. .T. [139]
JN831284_Thydatigena	C.G T..G. .T.G .G. T.T TAC .T. AT. ..A .A. [139]
AJ277409_Thydatigena	C.G TA.G. .T. .G. ..G .G. T.T TAC .T. AT. ..A .A. [139]
AJ277408_Thydatigena	C.G TA.G. .T. .G. ..G .G. T.T TAC .T. AT. ..A .A. [139]

Fig. 2. (continued)

remarkable, given its high zoonotic potential. In the last years, a screening of *E. multilocularis* infections in fox carcasses was carried out (Magi et al., 2015), along with a screening of gastrointestinal parasites in wolves (Gori et al., 2015), but no *E. multilocularis* infections were detected.

In our study area, there are several species of potentially competent IHs, and both dogs and wolves are known to host *E. multilocularis* (Romig et al., 2017). A previous study on wolf diet indicated predation upon small mammals with a frequency up to 7.6% in the same area (Imbert et al., 2016). Similarly, free ranging shepherd dogs are likely to be predators of these small mammals as well, although we do not expect that to be frequent. Despite that, it is not very likely that a stable *E. multilocularis* population could be maintained without the involvement of a DH species with a diet more focused on IHs such as the red fox. With one exception (China, Tibetan plateau (Moss et al., 2013; Vaniscotte et al., 2011)), there is no evidence that wolves and dogs may sustain the cycle.

For this reason, we have already started a collection of fox feces and organizing an opportunistic fox carcass interception in coordination with the local animal health agencies, but more in-depth studies are needed to assess the actual extent of the current distribution of *E. multilocularis* in this region and the possible origin of infections. Likely,

a larger screening of foxes along the Maritime Alps on both sides of the border (France and Italy) will be required. Moreover, since *E. multilocularis* positive dogs are expected to be present only in high endemic areas, our finding poses a potential threat for public health, and a screening of *Echinococcus* infections in dogs is strongly recommended.

This is the southern-most report of *E. multilocularis* in Italy, and the southern-most in Europe beside the most recent findings in Croatia and Serbia (Beck et al., 2018; Lalošević et al., 2016), and an older record from the European part of Turkey (Deplazes et al., 2017). This infection is likely due to a south-eastward expansion of the current *E. multilocularis* range in France.

Being a reportable disease, we recommend to start an epidemiological surveillance program to monitor the distribution of *E. multilocularis* in the Maritime Alps, along with an alert to the public health and veterinary professionals to warn about the possibility of AE in people and animals in the area.

The study of marginal populations of *E. multilocularis* offers a unique opportunity to explore the emergence of parasites with complex life cycles at the edge of their distribution, and explore the role of the host community in the establishment of new endemic areas. Moreover, monitoring these marginal populations is also of paramount importance to detect parasite range expansion and develop proper evidence-based

surveillance strategies and preventative measures.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ijppaw.2018.08.001>.

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