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Abstract: After centuries of massive decline, the recovery of the wolf (Canis lupus italicus) in Italy is a typical conservation success story. To learn more about the possible role of parasites in the wolves’ individual and population health and conservation we used non-invasive molecular approaches on fecal samples to identify individual wolves, pack membership, and the taeniids present, some of which are zoonotic. A total of 130 specimens belonging to 54 wolves from eight packs were collected and examined. Taeniid eggs were isolated using a sieving/flotation technique, and the species level was identified by PCR (gene target: 12S rRNA and nad1). Taeniid prevalence was 40.7% for Taenia hydatigena, 22.2% for T. krabbei, 1.8% for T. polyachanta and 5.5% for Echinococcus granulosus. The prevalence of E. granulosus is discussed. Our results show that the taeniid fauna found in wolves from the Foreste Casentinesi National Park is comparable to that described for other domestic and wild Italian canids and provides insights into the wolves’ diet and their relationship with the environment.

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Italian wolves (*Canis lupus italicus* Altobello, 1921) and molecular detection of taeniids in the Foreste Casentinesi National Park, Northern Italian Apennines

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**Article info**

**Abstract**

After centuries of massive decline, the recovery of the wolf (*Canis lupus italicus*) in Italy is a typical conservation success story. To learn more about the possible role of parasites in the wolves’ individual and population health and conservation we used non-invasive molecular approaches on fecal samples to identify individual wolves, pack membership, and the taeniids present, some of which are zoonotic. A total of 130 specimens belonging to 54 wolves from eight packs were collected and examined. Taenid eggs were isolated using a sieving/flotation technique, and the species level was identified by PCR (gene target: 125 rRNA and nad1). Taenid prevalence was 40.7% for *Taenia hydatigena*, 22.2% for *T. krabbei*, 1.8% for *T. polyacantha* and 5.5% for *Echinococcus granulosus*. The prevalence of *E. granulosus* is discussed. Our results show that the taenid fauna found in wolves from the Foreste Casentinesi National Park is comparable to that described for other domestic and wild Italian canids and provides insights into the wolves’ diet and their relationship with the environment.

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**1. Introduction**

After centuries of massive decline, several populations of large carnivores (brown bear, wolf, lynx, and wolverine) are now recolonizing parts of their historical ranges in many European countries thanks to the implementation of active adaptive conservation efforts (Chapron et al., 2014). The wolf in Italy is a typical conservation success story (Randi, 2011). At the end of the Second World War, Italian wolves were close to extinction, surviving at their historical minimum population size in two isolated areas in the Southern Apennines (Zimen and Boitani, 1975; Boitani, 1984, 1992). However, since the late eighties ecological changes and the increase in wild ungulates in natural areas have favored a spontaneous re-expansion of Italian wolves along the Apennines to the Western Italian and French Alps (Breitenmoser, 1998; Boitani, 2000; Valière et al., 2003; Fabbri et al., 2007; Marucco and McIntire, 2010). On one hand, the impact of this rapid recovery can increase conflicts with hunters seeking the same prey, livestock breeders suffering economic losses caused by wolf predation on domestic herds (Milanesi et al., 2015), and the general public many of whom have a historical fear of wolves, which are still perceived as a potential threat to human safety (Linnell and Boitani, 2011; Glikman et al., 2012). On the other, the wolf arouses positive harmonies as a flagship species whose biology, ecology and population dynamics remain poorly known in the Italian ecological context.

During the last 40 years, many studies have investigated the distribution and expansion of the Italian wolf population (Zimen and Boitani, 1975; Fabbri et al., 2007), its abundance (Marucco et al., 2009; Caniglia et al., 2012; Galaverni et al., 2016), composition and home ranges of packs (Ciucci et al., 1997; Apollonio et al., 2004; Scandura et al., 2011; Caniglia et al., 2014), its genetic variability (Randi et al., 2000; Randi and Lucchini, 2002; Lucchini et al., 2004), the threat posed by hybridization with domestic dogs...
A number of studies have investigated Italian wolf parasites (Arru et al., 1988; Guberti et al., 1991, 1993, 1998, 2004, 2005; Gori et al., 2015) because of the recognized role of wildlife parasites in shaping individual host fitness (Hudson, 2002) and their public health significance as zoonoses (Thompson, 2013). All these studies paid particular attention to Echinococcus granulosus sensu stricto (sheep strain genotype 1), an important emerging and re-emerging zoonotic agent, above all in the Mediterranean basin (Sadjadi, 2006). E. granulosus is a small tapeworm approximately 3 mm in length, endemic in this region since the appearance of sheep farming and hence a close relationship has developed over the centuries between the domestic dog (definitive host) and small ruminants as the main intermediate hosts. The official data of sheep cystic echinococcosis (CE) in Italy is summarized in Deplazes et al. (in press), while the prevalence in adult sheep is at least around 40% (Poglayen et al., 2008a, b). The low prevalence of cystic echinococcosis (CE) in wild ruminants, the main wolf prey, has prevented the establishment of a purely wild animal cycle so far (Guberti et al., 2004). The low number of wolves (n = 1300–1800) (Galaverni et al., 2016), a high prevalence of infected sheep (40%), and many positive dogs, allow the wolf to be considered in a parallel epidemiological context, closely linked to the domestic cycle (Guberti et al., 2004).

The other species of tapeworm give rise to speculation in attempts to understand and know more about the wolf diet, as the larval stage of each cestode has a specific host range (i.e. Taenia hydatigena: wild and domestic ungulates; T. krabbei: only wild ungulates; T. polyacantha: micromammals).

The aim of this molecular study was to evaluate the presence of taenid tapeworms in the wolves of the Foreste Casentinesi, Monte Falterona e Campigna National Park (FCNP), Northern Italy. This area provides opportunities to better understand the ongoing expansion of the Italian wolf population as some (Cagnolaro et al., 1974; Apollonio et al., 2004) claim that the wolf never disappeared from the FCNP, which acted as a natural ecological corridor along the Apennines guaranteeing the link between wolves from Central Italy and those of the Western Alps (Fabbri et al., 2007; Caniglia et al., 2014).

Most of the studies on wildlife intestinal parasites depend on standard methodologies based on post-mortem examination (Wobeser, 2007). As the wolf is a protected and elusive species these techniques are not a feasible option, so we used fecal analyses (Carbonell and Rodriguez, 1998) combining parasitological analysis with individual host genotyping based on fecal DNA (e.g. Zhang et al., 2011). This approach allowed us to identify each fecal sample’s taxonomic affiliation (e.g. wolf, dog or hybrid), genetic profile, sex and, thanks to the pedigree reconstruction, the family group to which it belonged (Lucchini et al., 2002; Fabbri et al., 2007; Marucco et al., 2012; Caniglia et al., 2014).

2. Materials and methods

2.1. Study area

The study area includes the Foreste Casentinesi, Monte Falterona e Campigna National Park (FCNP), Northern Italy (Fig. 1). From 2001 to 2008 the Environmental Section (CTA) of the Italian Forestry Corp (CFS) and Institute for Environmental Protection and Research (ISPRA) started an intensive genetic monitoring program based on the non-invasive collection of scat samples to investigate the presence, status and distribution of the wolf population in the FCNP. The project was carried out in the framework of a wider regional study, whose results are reported in Caniglia et al. (2014).

During the genetic monitoring project, 1433 non-invasive presumed wolf biological samples were collected in the FCNP and analyzed at the ISPRA Genetic Laboratory to identify the genetic profile of individual wolves. Feces were collected along trails or country roads chosen opportunistically to maximize the probability of finding fresh samples and covering the entire study area. Roads and trails were surveyed at least once per month and the geographic coordinates of every sample were recorded by GPS.

Small samples from the external portions of scats were individually stored in 10 vials of 95% ethanol. Before any manipulation,
as a safety precaution they were stored for 10 days at –80 °C (Eckert et al., 2001), then at –20 °C until DNA extraction.

DNA was extracted using the MultiPROBE Ilex robotic liquid handling system (Conquer Scientific, San Diego, CA, USA) and the QIAGEN QIAamp DNA stool extraction kit (QIAGEN Inc., Hilden Germany). The individual genotype of each sample was identified using a multiple-tube approach (Taberlet et al., 1997) at 12 unlinked autosomal canine microsatellites (short tandem repeats, STRs) selected for their polymorphism and reliable scorability, and a restriction fragment polymorphism to the ZFX gene to gender identification. Maternal haplotypes were identified by sequencing 350 base pairs of the mitochondrial DNA (mtDNA) control region and paternal haplotypes by typing 4 Y-linked microsatellites (Y-STR). DNA sequences and microsatellites were analyzed in a 3130XL ABI automated sequencer (Applied Biosystems, Foster City CA, USA), using the ABI software SEQSCAPE 2.5 for sequences and GENEMAPPER 4.0 for microsatellites (Applied Biosystems). GIMLET was used to reconstruct the consensus genotypes for each sample, compare them and control the good attribution of several samples to the same individual. The reliability of the reconstructed multilocus genetic profile was assessed using RELIOTYPE (Miller et al., 2002) and a threshold of 0.95. Only genotypes with a probability of reliability to >0.95 were retained. For details on PCR conditions and primer references, multi-tube protocol, reliability and match tests, see Caniglia et al. (2014).

2.3. Species identification from genetic profiles

We used STRUCTURE v.2.3 (Falush et al., 2003) to assign the individual genotypes as wolves, dogs, or wolf x dog hybrids. Reference wolf (n = 168) and dog (n = 160) genotypes were randomly selected from the ISPRA Canis database. We ran STRUCTURE with five replicates of 10^6 burn-in followed by 10^6 iterations of Markov chain Monte Carlo sampling, with the ADMIXTURE model and assuming independent allele frequencies. According to previous studies (Caniglia et al., 2014), the optimal number of populations was set at K = 2 (the value that maximized the posterior probability of the data). At K = 2, we assessed the average proportion of membership (Q) of the sampled populations to the inferred clusters. Then we assigned genotypes to the Italian wolf or dog clusters at threshold qi = 0.95 (individual proportion of membership; Randi, 2008), or identified them as admixed if their qi values were intermediate.

2.4. Pack identification and pedigree

We determined the spatial distributions by 95% kernel analysis using the ADEHABITATHR package for R (Calenge, 2006) for all the genotypes sampled in restricted ranges (<100 km²) at least four times and for periods longer than 24 months, and mapped them in ARCGIS 10.0. We performed parentage analyses considering candidate parents all the individuals sampled in the first year of sampling and more than four times in the same area, and candidate offspring all the individuals collected within the 95% kernel spatial distribution of each pack and in a surrounding buffer area of approximately 17-km radius from the kernel center (for details, see Caniglia et al., 2014).

2.5. Taeniidae identification

From the ISPRA genetic bank 130 specimens belonging to 54 wolves, chosen according to the genetic profile (not dog or wolf x dog hybrid) and to abundance of material, were examined for taeniid eggs.

Up to 2 g of feces were sieved in a filter (mesh 150 µm) and washed several times in a cup. The filtrate was centrifuged (1600 × g) for ten minutes and the pellet collected. Taeniid eggs were isolated from the pellet using the flotation and sieving method described by Mathis et al. (1996) and subjected to morphological identification under an inverted microscope. In egg positive samples and in 14 of the negative samples, DNA extraction was carried out with the complete sieving fraction as described by Štefanić et al. (2004). The 14 negative samples were included as negative controls. A total of 69 samples were analyzed using a multiplex-PCR to discriminate between E. granulosus and E. multilocularis and other cestodes including Taenia spp. (Trachsel et al., 2007). To obtain clear sequences of the E. granulosus positive samples, the PCR was repeated but only using E. granulosus primers (Cest5 and Cest4) keeping the same conditions as described above, and therefore amplicons were sequenced. Another PCR targeting nad1 gene (Armua-Fernandez et al., 2011) was used in samples without clear sequencing results to confirm the species of those samples. The amplicons were directly sequenced after purification of the PCR products using the MinElute® PCR purification kit (Qiagen). Sequencing was performed by Syngene Biotech GmbH, Biotech Center Zurich, Switzerland (http://www.syngene-biotech.com). Primer Cest5 and Cestseq was used for non-Echi- nomorpha cestodes including Taenia spp. and Cest5 for E. granulosus, while primer nad1T-Rv for Taenia spp. for amplicons obtained with multiplex-PCR and nad1 PCR, respectively. Sequences were compared with the one present in GenBank using Blast tool (http://www.blast.ncbi.nlm.nih.gov).

3. Results

3.1. Genetic individual identification and pack reconstruction

From the 1433 analyzed samples, 544 were successfully genotyped (38%), belonging to 137 individuals: 117 wolves and 20 dogs (no wolf x dog hybrids were identified). The kinship and spatial analyses identified eight packs within the FCNP park (Fig. 2a), for which complete genealogies were reconstructed and are available in Caniglia et al. (2014).

3.2. Parasite identification

We examined 130 fecal samples from 54 different wolf individuals, of which 35 individuals (and 90 corresponding samples) belonged to the eight packs (Fig. 2) and 19 were not assigned to any known pack (40 samples). Fecal samples were examined only for Taeniidae eggs, and showed a 42.1% frequency (55/130) and a prevalence of 61.1% (33/54) (Tables 1 and 2, respectively). Among the five parasite species isolated, T. hydatigena was the most common in terms of frequency in all samples (23.8%) and prevalence in the population examined (40.7%), followed by T. krabbei with a frequency of 10.7% and a prevalence of 22.2%. One sample (0.7%) corresponding to one animal (1.8%) was positive for T. polycauncta. E. granulosus “sensu stricto” (G1–G3) was found in three samples (2.3%) belonging to three different wolves (5.5%).

The nucleotide sequence was not obtained from six positive samples, so the taeniids could only be identified as other cestodes including Taenia spp., while no sample was positive for E. multilocularis. Of the 33 positive wolves, 22 were sampled once (66.7%), five twice (15.1%) and of these one only was positive for the same parasite (T. krabbei) in the second sampling. Four wolves were sampled three times (12.2%) and three of these maintained positivity for T. hydatigena. One wolf (3%) was sampled four times and another five times. Two samples were simultaneously positive for T. hydatigena and E. granulosus (Fig. 2b).
4. Discussion

Our 130 fecal wolf samples showed a taeniid prevalence close to 60%, the most common being *T. hydatigena* with a prevalence of 40.7%. None of the eight family packs presented the same composition of taeniid fauna, and only one had all four isolated species. As expected, since no sample was positive for *E. multilocularis*, the prevalence of the other zoonotic cestode, *E. granulosus* (G1-G3), is not surprising because of its wide diffusion in Italy. In fact, when slaughterhouse data were matched with the national ovine registry to identify the geographical origin of animals all over the country, CE prevalence was at least 40% in adult sheep (Poglayen et al., 2008a,b). The lower detection rate compared with Gori et al.’s findings (2015) should be ascribed to the particular environment of our wolves, a national park with a high wild prey density and virtually echinococcosis-free. No wild cycle of *E. granulosus* has been described in Italy and the *E. granulosus* prevalence in carnivores in this park is probably linked to predation on domestic animals. The information on wolf attacks stems from a Regional program to refund damaged breeders and thereby contribute to wolf conservation. These attacks appear very close to the park boundaries with three cases even inside a wolf pack (Fig. 3). The presence of *E. granulosus* in wolves far away from the reported attacks could be attributed to the wolves’ mobility also to reduce energetic hunting efforts. According to Guberti et al. (2005), the low prevalence of *E. granulosus* is further confirmation of the absence of a wild cycle of this parasite. A deterministic model was adopted to simulate a purely theoretical sylvatic cycle and demonstrate that even if both the wolf and the wild ungulate population are increasing, the wolf is still part of the parasite’s main dog/sheep

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**Table 1**

| Total samples n = 130 | Taeniid species                  | Number of positive samples (Frequency %) | Confidence interval (95%) |
|-----------------------|----------------------------------|------------------------------------------|---------------------------|
| *Taenia hydatigena*   | 31 (23.8)                        |                                          | 16.5–31.1                 |
| *Taenia krabbei*      | 14 (10.7)                        |                                          | 5.4–16                    |
| *Taenia polycanthera* | 1 (0.7)                          |                                          | 0.0–2.1                   |
| *Echinococcus granulosus* (G1-G3) | 3 (2.3)                       |                                          | 0.0–4.8                   |
| non-Echinococcus cestodes including Taenia spp. | 6 (4.5)                        |                                          | 1–8.2                     |
| **Total**             | **55 (42.1)**                     |                                          | **33.7–50.5**             |

**Table 2**

| Total wolves n = 54 | Taeniid species                  | Number of positive animals (Prevalence %) | Confidence interval (95%) |
|---------------------|----------------------------------|------------------------------------------|---------------------------|
| *Taenia hydatigena* | 22 (40.7)                        |                                          | 27.6–53.8                 |
| *Taenia krabbei*    | 12 (22.2)                        |                                          | 11.2–33.2                 |
| *Taenia polycanthera* | 1 (1.8)                        |                                          | 0.0–5.3                   |
| *Echinococcus granulosus* (G1-G3) | 3 (5.5)                        |                                          | 0.0–11.5                  |
| non-Echinococcus cestodes including Taenia spp. | 6 (11.1)                       |                                          | 2.8–19.4                  |
| **Total**           | **33 (61.1)**                     |                                          | **48.1–74.1**             |
cycle (Guberti et al., 2004). To confirm this stochastic model, an active surveillance program on wild fauna hunted or found dead in our region has been implemented by public research laboratories (Istituto Zooprofilattico Sperimentale) with the main aim to protect livestock. No CE has ever been detected in the wild ruminants of the area (Tosi pers comm).

The presence of taeniids is always related to the host diet. Although the wolf is a carnivore, its diet is varied and well suited to the different trophic niches offered in southern Europe where this canid has apparently adapted to feed on fruit, rubbish and livestock as well as small and medium-sized mammals (Meriggi and Lovari, 1996). The lack of specific surveys on parasite fauna in different potential prey means we can only speculate on the presence of metacestodes.

*T. hydatigena* was the most common species detected in stools and wolves in our survey. This parasite is also common in wolves in Europe (Craig and Craig, 2005; Moks et al., 2006; Bagrade et al., 2009; Ćirović et al., 2015) whose intermediate hosts belong to wild and domestic ungulates. The second commonest parasite species was *T. krabbei* whose lower presence should be linked to a strictly wild cycle as the main intermediate host and wolf prey in the FCNP is roe deer. Before the advent of molecular biology tools, *T. ovis* and *T. krabbei* were difficult to distinguish morphologically, Lavikainen et al. (2008) suggested possible identification mistakes in old studies which may have included the isolation of *T. ovis* in Italy (Guberti et al., 1993). *T. krabbei* is also common in Europe (Craig and Craig, 2005; Bagrade et al., 2009). *T. polyacantha* reflects the presence of intermediate hosts (micromammals), and its low prevalence (1.8%) suggests wolves make scarce use of these kinds of prey. According to Scaravelli (2001), 19 micromammal species are present in the FCNP.

Our study differs from the other six national studies on taeniids. One adopted an epidemiological approach (Gori et al., 2015), while the other five were both parasitological and epidemiological (Guberti et al., 1991, 1993, 1998, 2004, 2005) and referred to 119 dead wolves collected throughout the Apennines range of species distribution (Table 3). Therefore the results were obtained by total worm count followed by morphological identification of parasites. The only possible comparison between these and our data is in terms of taeniid presence/frequency. More recently, Gori et al. (2015) reported the molecular results for cestodes from 179 fecal samples attributed to wolf, evaluating size, shape, smell and composition according to Bassi et al. (2012) in the Liguria region. In this case, the same parasitological approach was adopted, but lacking the species identification from the genetic profile the stool recognition is less accurate: in fact, stray dogs in the Emilia-Romagna region are confined in kennels, whereas in Liguria many stray dogs are still free-ranging. This would create some bias on the actual species sampled. Furthermore, the Liguria study area is larger and included the whole region (540,000ha) where scattered protected and hunting areas are mixed, whereas our area includes only a small National Park (36,000ha). The comparison with last survey can be done only in term of taeniids frequency (Table 3). In our surveys, each scat is molecularly referred to a single wolf of a single pack (Fig. 2a).

In summary, the main differences between previous national experiences are by parasitological and epidemiological approach with Guberti et al. (1991, 1993, 1998, 2004, 2005; Gori et al., 2015). The only data from the same area on carnivore taeniids, isolated by necropsy, referred to foxes in which taeniid species were the same (Poglayen et al., 1985, 1988; Fiocchi et al., 2016).

From a public health perspective, it is important to emphasize the absence of *E. multilocularis* in the Apennines. In recent years, this taeniid has become an important parasite in Northern Europe, also in an urban context. The only stable small focus is present in foxes of North-Eastern Italy with no human cases (Casuli et al., 2005; Dellamaria et al., 2014).

Among the taeniids detected, our study focused on *E. granulosus*. In Italy, CE is widely prevalent in livestock (Garioppa, 2006; Deplazes et al., in press), making wolf infection a negligible aspect in the public health context. Efforts to combat CE target the domestic cycle using well-known tools of proven efficacy. National abattoirs ensure the destruction of positive offal so that no infectious material may enter the meat production cycle. The problem arises with frequent illegal home slaughter which favours the spread of the parasite among shepherds and farm dogs and contributes to

| Taeniids               | Gori et al. (2015) | Present study |
|------------------------|-------------------|---------------|
| *Taenia hydatigena*    | 19.6              | 23.8          |
| *Taenia krabbei*       | 4.5               | 10.7          |
| *Taenia ovis*          | 2.2               | Not found     |
| *Taenia crassiceps*    | 0.6               | Not found     |
| *Hydatigena taeniaformis* | 0.6     | Not found     |
| *Echinococcus granulosus s.l.* | 5.6 | –             |
| *Echinococcus granulosus s.s.* | –    | 2.3           |
| *Taenia polyechanta*   | Not found         | 0.7           |

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Fig. 3. Distribution of attacks on livestock in the Emilia-Romagna region, near and inside the FCNP wolf packs. (Data from Emilia-Romagna attacks control program.)
maintain high infection rates in these dogs, ruminants and humans. The lack of a national CE control program is solely responsible for the maintenance of the disease in these regions. The lack of a national CE control program is solely responsible for maintaining high infection rates in these dogs, ruminants and humans. Therefore, the combined approach involving collaboration with veterinarians and pathologists was used to establish the parasitological expertise. Nature is a complex mosaic and in-depth study of one tile does not shed light on the whole picture.

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The combined non-invasive method adopted in this study confirms its importance in the study of ecology, behavior and parasitology without interfering with the sensitive population dynamics of Italy’s most important carnivore. In addition, our global approach involved collaboration with veterinarians and pathologists in the Italian wolf population sharing our parasitological expertise. Nature is a complex mosaic and in-depth study of one tile does not shed light on the whole picture.
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