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The Eastern cottontail (Sylvilagus floridanus) in Tuscany (Central Italy): weak evidence for its role as a host of EBHSV and RHDV.

Type

Research paper

Keywords

Eastern cottontail, Sylvilagus floridanus, RHDV, EBHS, EBHSV, lagovirus

Abstract

During the last few decades native European hares (Lepus europapeus) have declined in Central and Northern Italy. Despite this trend having multiple causes, it was hypothesized that invasive Eastern cottontails (Sylvilagus floridanus) contributed to the decline through apparent competition and disease transmission. In this research we explored whether cottontails may act as carriers of EBHSV (European Brown Hare Syndrome Virus) and RHDV (Rabbit Haemorrhagic Disease Virus), the viral agents of two major diseases affecting lagomorphs in Europe. We took biological samples from 267 cottontails that were shot between March and August 2015 in Tuscany, performing specific antigenic and serological ELISA tests for both viruses as well as molecular investigation for lagoviruses. Virologic tests were all negative and serological titers were below the threshold that could indicate the active circulation of either of the two pathogenic viruses. Our findings suggest that cottontails were not playing an active role as carriers or reservoirs for both known virulent lagoviruses and were also not infected with non-pathogenic lagoviruses - at least at that time in the study area.

Explanation letter

Minor revisions for the final draft, as suggested by the Editor, were implemented.

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Title: The Eastern cottontail (*Sylvilagus floridanus*) in Tuscany (Central Italy): weak evidence for its role as a host of EBHSV and RHDV.

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Running title: Invasive cottontails and the EBHSV and RHDV.

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Abstract

During the last few decades native European hares (*Lepus europaeus*) have declined in Central and Northern Italy. Despite this trend having multiple causes, it was hypothesized that invasive Eastern cottontails (*Sylvilagus floridanus*) contributed to the decline through apparent competition and disease transmission. In this research we explored whether cottontails may act as carriers of EBHSV (European Brown Hare Syndrome Virus) and RHDV (Rabbit Haemorrhagic Disease Virus), the viral agents of two major diseases affecting lagomorphs in Europe. We took biological samples from 267 cottontails that were shot between March and August 2015 in Tuscany, performing specific antigenic and serological ELISA tests for both viruses as well as molecular investigation for lagoviruses. Virologic tests were all negative and serological titers were below the threshold that could indicate the active circulation of either of the two pathogenic viruses. Our findings suggest that cottontails were not playing an active role as carriers or reservoirs for both known virulent lagoviruses and were also not infected with non-pathogenic lagoviruses - at least at that time in the study area.

Introduction

In the last few decades populations of European hare (*Lepus europaeus*) have faced a widespread decline across many European countries (Smith et al. 2005). This decline has been probably caused by a combination of changes in the environmental quality of agricultural ecosystems, overharvesting, wrong restocking schemes, increased predation rates and infectious diseases (Pavliska et al. 2018; Schai-Braun et al. 2015; Schmidt et al. 2004; Swinton et al. 2002). Among diseases, it is worth noting that European Brown Hare Syndrome Virus (EBHSV), a highly viral disease caused by a lagovirus (Caliciviridae family) has been known to affect hares since the late 1980s (Poli et al. 1989) and has become a major source of mortality for European populations (Frolich and Lavazza 2007). Moreover, recently the “new” Rabbit Haemorragic Disease Virus (RHDV) type 2 (RHDV2), another highly virulent lagovirus, was also found to infect and cause disease in at least four different species of hares (Camarda et al. 2014; Neimanis et al. 2018; Puggioni et al. 2013; Velarde et al. 2017), while the “classical” RHDV, the prototype of the lagovirus genus, was deemed to only affect wild rabbits (*Oryctolagus cuniculus*) (OIE Rabbit Haemorragic Disease, 2012), with just a single exception reporting two Iberian hares collected in the 1990s in Portugal (Lopes et al. 2014). Hare decline in Europe has also occurred in Central and Northern Italy (Santilli and Galardi 2016; Santilli 2007), where both RHD and EBHS should be considered endemic since late 1980s. In these areas, invasive Eastern cottontails (*Sylvilagus floridanus*) were introduced in the 1970s, and they are still expanding their geographical distribution due to illegal restocking for recreational hunting (Cerri et al. 2016). Cottontails do not seem to compete directly with native hares, as the two species select different habitats (Vidus-Rosin et al. 2011). However, cottontails could affect prey-predator dynamics between native hares and red foxes (*Vulpes vulpes*) (Cerri et al. 2017). Indeed, cottontails were found to carry a wide range of parasitic and fungal diseases (Bertolino et al. 2010; Gallo et al. 2015; Zanet et al. 2013), and it was hypothesized they could have a role as carriers of EBHSV or as a possible host of other lagoviruses, such as non-pathogenic Rabbit caliciviruses (RCVs) (Capucci et al. 1996; Strive et al. 2009) and Hare Calicivirus (HaCV) (Cavadini et al. 2015; Lemaitre et al. 2018) that could potentially evolve to virulent RHDV strains through a mechanism of species jump (Esteves et al. 2015). Notably, Lavazza et al. (2015) found that cottontails could be infected with the EBHSV, both naturally and in a laboratory environment, and that cottontails from Central and Northern Italy had specific EBHSV antibodies. Therefore, cottontails could hypothetically play a role as carriers or reservoirs for lagoviruses involved in the large-scale population decline of the European hare in Central and Northern Italy.

The main aims of this research were: i) to obtain further evidence for the potential role of invasive Eastern cottontails in supporting the replication and active circulation of virulent lagoviruses (EBHSV and RHDV/RHDV2) and ii) to detect in *Sylvilagus* the presence of possible non-pathogenic lagoviruses, similar to RCV of rabbits and HaCV of hares. To do that, we collected samples from cottontails living in Tuscany, an area where the species is quickly expanding its geographical range and locally reach very high densities (Cerri et al. 2016), and where both EBHS and RHD have been continuously reported in native European hares and wild rabbits (Poli et al.,1991; Lavazza et al. 2013), since 1990s.

Materials and Methods

Samples were collected in 2015, from March to August (Table 1). The study area (Figure 1) was in Castelmartini, in the Tuscany Region (43°49'21,4" N; 10°49'51,9" E). It encompassed a hunting estate of 500 hectares where hares are normally present and cottontails reach a density of about 50 individuals/km^2. In the area there are no wild rabbits since the nearest know population is at least 20 km far from there. Samples were collected from animals that were shot with a firearm during control schemes authorized by the National law about wildlife control (art. 19, law n. 157/92). Cottontails were sexed through visual assessment of the genitalia.

Sandwich ELISA tests (ELISA-Ag) were adopted to search for EBHS and RHD-related antigens is specific tissue samples, and competition ELISA (cELISA) tests were adopted to look for specific EBHSV and RHDV antibodies in blood serum samples. These methods are described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE Rabbit Haemorrhagic Disease, 2012).

For the first sampling in March 2015, blood was collected from a total of 240 cottontails by means of blotting paper, as suggested by Portejoie et al. (2009), and about 10% of carcasses (n=23) were selected (one every ten animals shot) and immediately frozen, for further analysis. Carcasses were also chosen according to their integrity, as shooting often damages organs. Blood samples on blotting paper were eluted according to the method described by Portejoie *et al*. (2009) and adapted by Chiari *et al*. (2012b). Briefly, for each cottontail a small square of approximately 6x6mm was cut from each of two dried blotters and both pieces were placed separately in 100 µl of phosphate-buffered saline (pH 7,4), held overnight at 4°C and then 32 µl of the eluted solution was recovered for cELISA tests for EBHSV and RHDV. The first dilution (1:2) corresponds to 1:10 dilution of serum and thus the serum equivalent titre was obtained by 5x multiplication of the titre obtained with the eluted solution from two discs of blotting paper. The twenty-three carcasses were subjected to necropsy and, independently from the detection of specific lesions referable to lagovirus infection, the liver, spleen, and part of the intestine (duodenum) were removed and analyzed with ELISA-Ag and molecular methods (RT-PCR).

In the second sampling in July-August 2015, 27 cottontails were tested. Immediately after a cottontail was shot its blood was collected directly from the open wound using a sterile single-use syringe, transferred into Vacutainer tubes and kept refrigerated. Blood samples were then centrifuged to separate the serum, which was placed in Vacutainer tubes and frozen until delivery to the diagnostic laboratory for being analysed by cELISA tests for EBHSV and RHDV. During necropsy part of the duodenum and a piece of liver and spleen were sampled and immediately frozen for being tested in ELISA-Ag for RHDV and EBHSV antigens.

For detecting lagoviruses in the duodenum, which is the recognized site of replication of the non-pathogenic RCVs and HaCV, we used the One Step RT-PCR kit (Qiagen) with the universal primers for lagovirus Rab1/Rab2 (Strive et al. 2009), and/or with the primers HaCV-F/HaCV-R (Cavadini et al. 2015).

We compared the proportion of positive samples between blotting paper and blood serum both for EBHSV and RHDVs, through two-tailed z-test for proportions, considering thatthe cELISA test is characterized by a fixed sensitivity and specificity (not affected by the matrix: blotting paper or blood serum).

Results and Discussion

The results of the cELISA tests (Table 1) show that out of 240 blood samples eluted from blotting paper (first sampling run), 40 (16.7%) were positive for EBHSV antibodies and 66 (27.5%) were positive for RHDV antibodies; indeed, 17 were the samples positive (7.1%) for antibodies against both RHDV and EBHSV. Thirty-nine samples that were positive for EBHSV had an antibody titer of 1/10 and one sample had a value of 1/20. Sixty-one samples that were positive to RHDV had an antibody titer of 1/10, three samples had a titer of 1/20, one sample had a titer of 1/40 and one sample had a titer of 1/80. Fourteen out of 17 samples positive for both viruses had the same titre (1/10), two samples had higher titres for RHDV (1/80 and 1/20) than EBHV (1/10) and one had a titre higher for EBHSV (1/20) than for RHDV (1/10). By using blood serum from the 27 shot animals (second sampling run) we found results, in terms of antibodies prevalence and titre value distribution, almost similar but not identical, to those obtained by using blotting paper. In particular, 6 individuals (22.2%) were positive to cELISA for EBHSV antibodies (all with titre 1/10) and 5 (18,5%) for RHDV antibodies (1/10); no samples were positive for antibodies against both viruses. The proportion of positive samples did not differ between blotting paper and blood serum, either for EBHSV (χ^2 = 0.21, df = 1, p-value = 0.65), or for RHDV (χ^2 = 0.60, df = 1, p-value = 0.44).

According to Chiari *et al.* (2012b), alternative sampling methods such as blotting paper and heart clots only predict 60% of the antibody titres obtained from sera. In this research, stating that the low sampling numbers do not permit to make a true analytical comparison, this moderate underestimation of titres did not undermine the interpretation of sero-epidemiological results. Our results were characterized by an almost overlapping prevalence and titres in the two different matrixes, and blotting paper samples were almost 9 times more numerous than sera, compensating for the imperfect detection of antibodies. Taken together, these results indicate that blotting paper is an alternative sampling method that can be extremely useful for lagovirus field studies.

From the 50 necropsied carcasses, testing of the liver and spleen samples by ELISA-Ag and duodenum samples by RT-PCR provided no antigenic and genomic positivity for lagoviruses. Our findings were negative both for virulent viruses (EBHSV and RHDV) as well as for non-pathogenic ones (RCVs-like and HaCV), indicating that the examined cottontails in the study area during spring-summer 2015 were not actively infected by any lagovirus.

The lack of detection of pathogenic lagoviruses in cottontails was not surprising. Apart from the sporadic occurrence of EBHSV in cottontails and the unproven susceptibility of cottontails to RHDV (Lavazza et al. 2015), it would be rare to find the viruses in the target organs of healthy lagomorphs shot during control schemes or killed during the hunting season (Cammi et al. 2003). Indeed, the real novelty was the lack of detection of any non-pathogenic viruses. Since RCVs and HaCV are quite commonly found in the other lagomorph species, like wild rabbits or the European hare, the possible existence of an analogous non-pathogenic virus in cottontails has been postulated (Esteves et al. 2015) and also largely investigated, but till now with negative results (Le Gall, Cavadini, Bertagnoli, personal communication from the ANIHWA-ECALEP project).

Moreover, in the 267 tested individuals the overall serological prevalence for both EBHS (46 positive = 17.2%) and RHDV (71 positive = 30.7%) was relatively low and largely different, in terms of both prevalence and titre distribution from figures normally found in rabbits and hares in areas where respectively RHD (Cooke et al., 2000; Mutze et al., 2014) and EBHS (Cammi et al. 2003; Chiari et al. 2012a) are endemically present. In fact, even if with a certain density-dependent variability, prevalence in EBHS and RHD endemic regions, like the study area, could be as high as 70-90% with medium-low titres (1/40-1/640). Indeed, the serological results of this study are very similar to those found in previous surveys (Lavazza et al. 2015) on cottontails conducted in North-Central Italy, when overall seroprevalences of 17.9% and 33.7% were observed for EBHSV and RHDV antibodies, respectively. In particular the prevalence for EBHSV antibodies in European hares, during the period 2003- 2012 in nine provinces, including also Firenze and Pistoia in Tuscany, was 20.1%. However, differently from those results where a number of cottontail sera exhibited high titres for EBHSV (i.e., up to 1/1280), most sera we examined had low titres (range 1/10-1/80), just above the threshold value (1/10). This is very different to those normally found in convalescent rabbits and hares which have suffered from clinical disease i.e. usually >1/640-1/1280 up to 1/20000 (Drews et al. 2011; Zanni et al. 1993).

Considering that all the samples tested by PCR for non-pathogenic lagoviruses were negative, it is harder to explain the origin of such serological “signal”, obtained by using specific serological methods like cELISAs. Apart from a nonspecific reaction of the sera, a hypothesis that cannot be totally ruled out, we might hypothesize that a limited number of cottontails had been infected months before our tests and therefore their serological status was characterized by a decreased low level of antibodies. Another potential explanation could lie in the infection of cottontails with an unknown non-pathogenic lagovirus, which might be able to induce cross-reactive antibodies that were partially detected by the RHDV and EBHSV cELISA tests. Moreover, the existence of common epitopes on all lagoviruses could be the explanation of a low reactivity, close to the threshold value (1/10), found for few sera (17 = 6.4%) in both cELISA for RHD and EBHS antibodies.

Finally, even though we did not specifically investigate the occurrence of RHDV2 virus, we are confident that any disease or infection of *Sylvilagus* with RHDV2 would have been detected by our virological tests. In fact, one of the RT-PCR methods here used employs universal primers for lagovirus Rab1/Rab2 (Strive et al. 2009) able to detect either virulent and non-pathogenic lagoviruses.

As RHDV2 has been found to infect hares (Camarda et al. 2014, Neimanis et al. 2018; Puggioni et al. 2013; Velarde et al. 2016), it deserves further attention as it might be able to infect *Sylvilagus* better than RHDV1.

Although we did not carry out random sampling, and therefore we did not make any inference about cottontail population as a whole, our findings are highly suggestive of the absence of any active circulation of lagoviruses in cottontails examined in this study. During spring-summer 2015, in our study area, cottontails appear not to have been a reservoir or occasional host for both EBHSV and RHDV as well as non-pathogenic lagoviruses. However, we suggest future studies should also use specific tests for RHDV2 antibodies. In addition, we intend to extend our approach to a broader geographical area, including all the various subpopulations of Eastern cottontails occurring in Central and Northern-Italy. Since cottontails were recently reported in the Latium region, close to the geographical distribution of the native Corsican hare (*Lepus corsicanus*, Dori et al. 2018), we suggest monitoring the occurrence of lagoviruses in these populations.

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Table 1. Summary of serological results: N° of positive (prevalence %) and [range of antibody titres]

Samples type	N° samples	N° tot pos		
		EBHS cELISA	RHD cELISA	EBHS & RHDV cELISA
Blood elution from blotting paper	240	40 (16.7%) [1/10-1/20]	66 (27.5%) [1/10-1/80]	17 (7.1%) 14 RHDV = EBHSV 2 RHDV > EBHSV 1 EBHSV > RHDV
Blood serum from fresh carcasses	27	6 (22.2%) [1/10]	5 (18.5%) [1/10]	0
Total	267	46 (17.2%)	71 (26.6%)	17 (6.4%)

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Figure 1. Map of the study area: boundaries of the hunting estates where cottontails were shot and its location in Italy.

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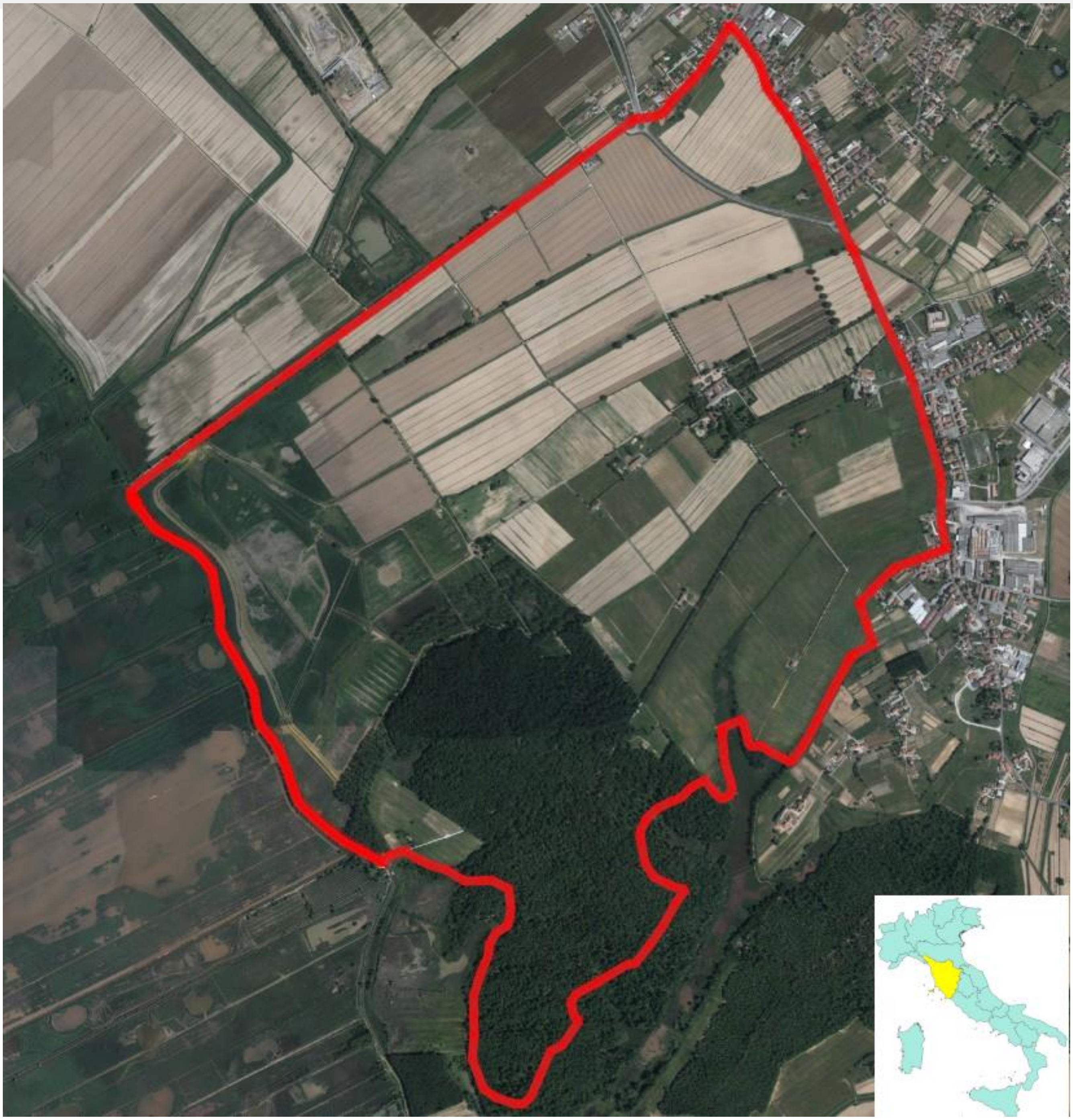

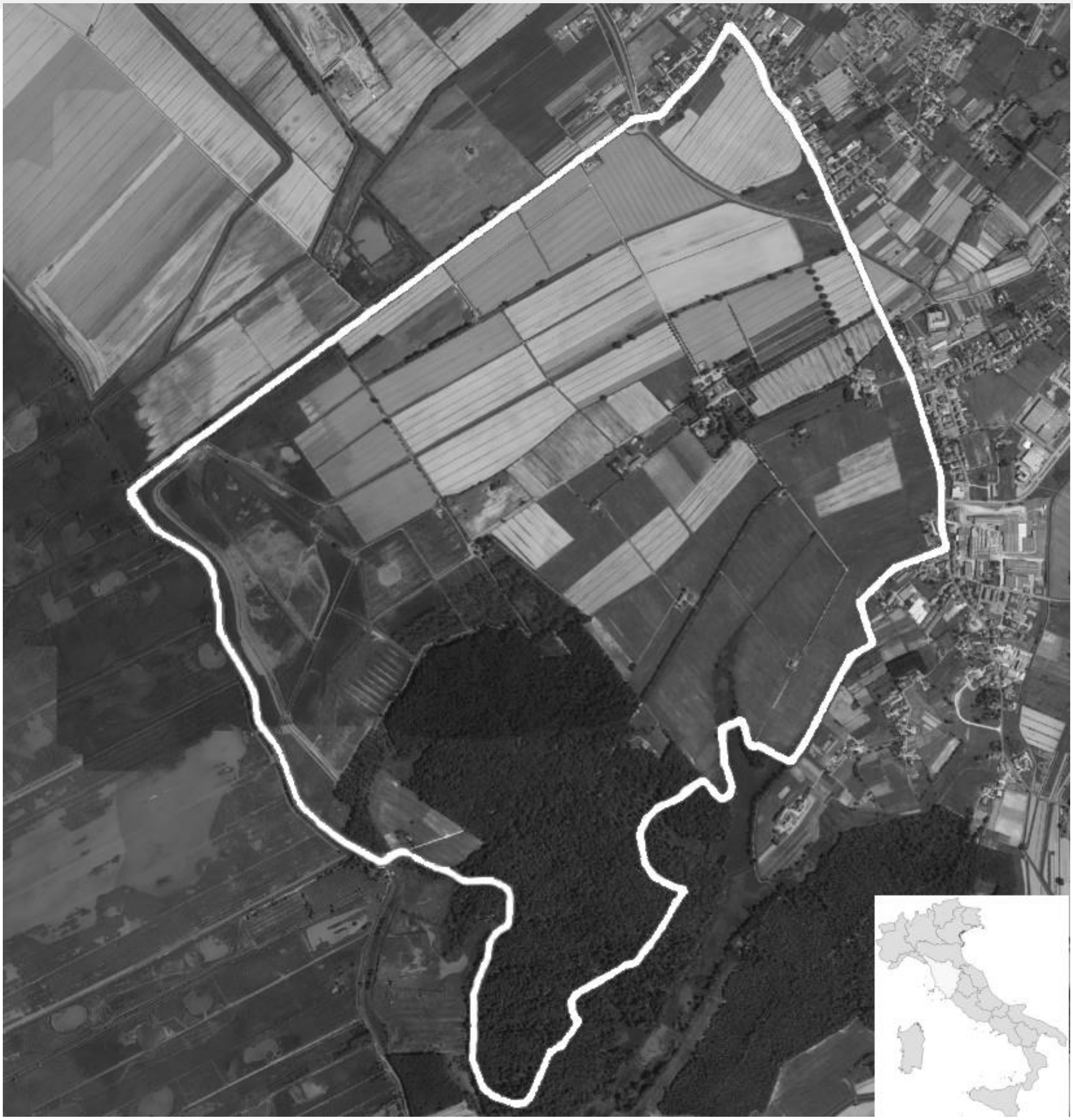

Figure 1
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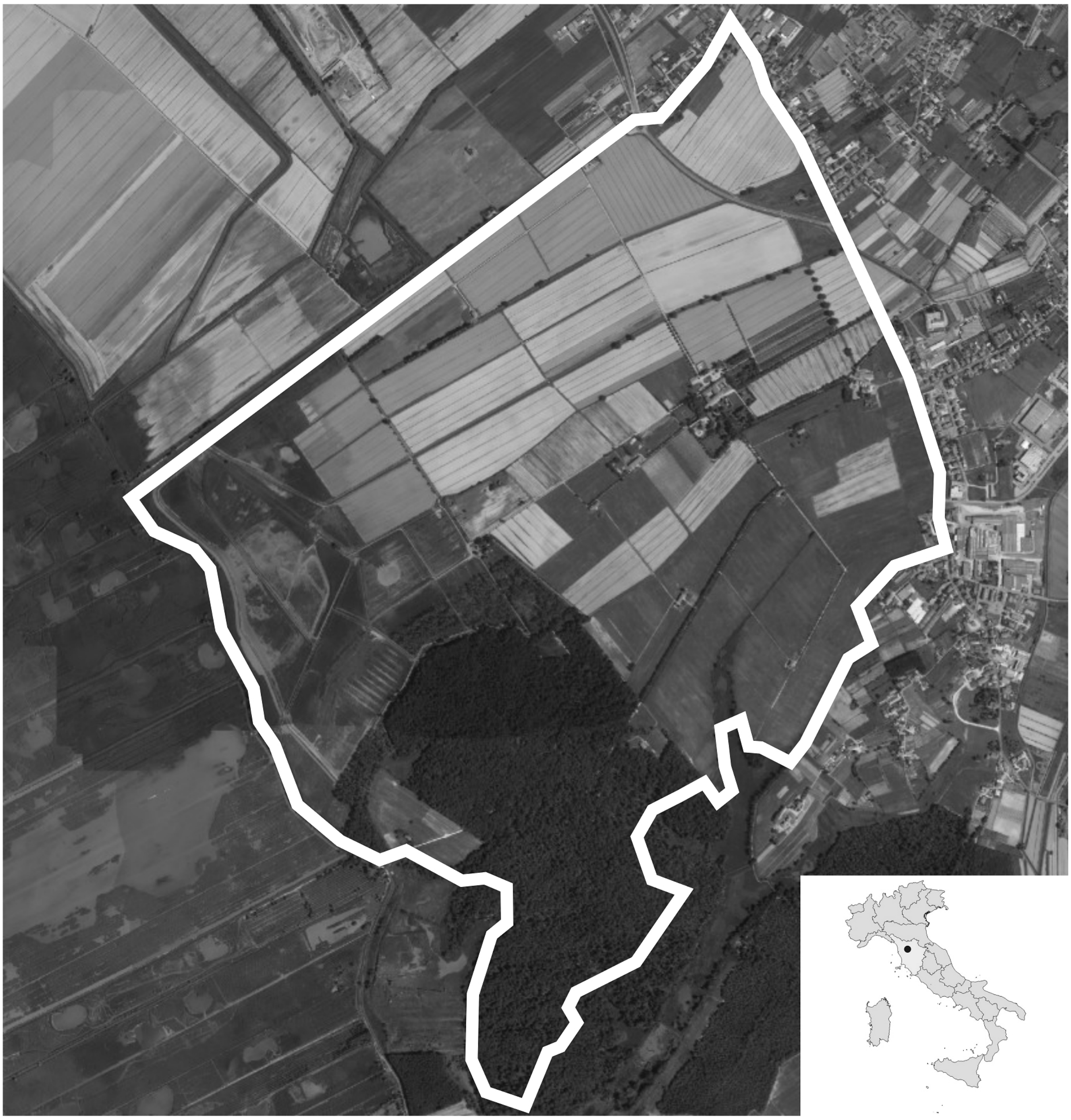

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