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The role of wild boars in spore dispersal of hypogeous fungi

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Wild boars (*Sus scrofa* L.) are well-known for soil disturbance in natural and cultivated truffières but their role in spore dispersal is poorly investigated. In the present work we studied the occurrence of hypogeous fungal spores in faecal contents of 14 wild boars randomly hunted in "Parco dei Gessi e Calanchi dell'Abbadessa" Regional Park (North of Italy) where truffle production has been previously investigated for three years.
Six methods for spore analysis in faeces were compared and the suspension of faeces in $ZnSO_4$ (70%) solution resulted to be the most reliable.
Hypogeous fungal spores, including *Tuber magnatum* and *Tuber aestivum* spores, were detected in 9 animals. This result suggests that the detection of fungal spores in faeces of wild boars may provide information on the presence of hypogeous fungi in an area. However, the poor abundance of spores suggests that the wild boar can be considered an opportunistic mycophagist, ingesting truffles only occasionally, as a seasonal source of food. Considering the magnitude of wild boar movements during seasonal migrations, it is possible to speculate that they play a key role in truffle long distance dispersal.

Key words: *Sus scrofa,* truffles, mycophagous, spore dispersal, faeces

INTRODUCTION

Hypogeous fungi comprise species belonging to several genera of Ascomycota (true truffles), Basidiomycota and Muromycotina (false truffles) (Trappe et al. 2009; Bonito et al. 2013). The hypogeous lifestyle comported several morphological changes, as the loss of the ability to discard spores actively (Trappe, Claridge 2005). This led to develop several survival strategies, as the development of a strong aroma, even typical of any

truffle species (Gioacchini et al. 2005). This aroma is useful to the fungus to be found and eaten by wild animals, mainly rodents and mammals, ensuring spore dispersal into the environment by animal faeces (Cazares et al. 1999; Trappe, Claridge 2005).

Indeed, faeces examination of mycophagous rodents, such as the giant white-tailed rat, *Uromys caudimaculatus* Krefft, the golden mantled ground squirrel, *Spermophilus saturates* Rhoads, and the deer mouse, *Peromyscus maniculatus* Wagner, demonstrated that truffles represent a consistent part of their diet (Comport, Hume 1998; Cork, Kenagy 1989).

Among mammals, wild boars (*Sus scrofa* L.) are sadly known to damage truffle production in natural and cultivated truffières (Ricci 2008; Moreno-Arroyo et al. 2005). The negative impact of wild boars on truffles is attributed to soil disturbance and ascoma consumption, as we demonstrated for *Tuber aestivum* in natural truffières in Central Italy (Salerni et al. 2011).

However, less is known on the possible ecological role of wild boars in truffle spore dispersal (Genard et al. 1986; Steiner, Fielitz 2009). As far as we know, only the former Authors reported specifically about *T. aestivum* consumption by wild boars, but the paper lacks in technical information about spore isolation methods.

Thus, in the present work we investigated the occurrence of hypogeous fungal spores in faecal contents of wild boars hunted in "Parco dei Gessi e Calanchi dell'Abbadessa" Regional Park, an area known for truffle production.

MATERIALS AND METHODS

Study area. The study was carried out in "Parco dei Gessi Bolognesi e Calanchi dell'Abbadessa" (Ente di gestione per i Parchi e la Biodiversità - Emilia Orientale, 2013) which is located in the South Eastern hilly area of Bologna (Central Italy) in the Municipalities of Bologna, Ozzano dell'Emilia, Pianoro and San Lazzaro di Savena (surface area 4.815,87 ha).

In this area, the forests are mainly represented by *Ostrya carpinifolia* Scop. and *Quercus pubescens* Willd. (all. *Orno Osrtryon* Auct. Ital. and all. *Ostryo-Carpinion orientalis*, Horvat 1954) (Corbetta 1994). The park is characterized by a temperate Mediterranean climate (Köppen-Geiger classification), where the highest and lowest temperatures occur in July-August and December-January, respectively, and the annual average precipitation is 750 mm (Pieri et al. 2011). Truffles, in particular *T. aestivum* and *T. magnatum,* are principally found in *Q. pubescens* mixed forests, in calcareous soils derived from marnous arenaceous rocks, within the Municipalities of Ozzano dell'Emilia, Pianoro and San Lazzaro di Savena.

Hypogeous sporoma collection. Fruiting bodies of truffles were collected in *Q. pubescens* forests of the Park, using trained dogs, between September and January 2004-2007.

Animals. Between October 3rd and December 4th 2011, the faeces of 14 wild boars hunted in the Park area, in compliance with the permitted hunter-kill ratio actions established by Bologna Province, were examined. Animals, randomly chosen regardless sex, age and weight, were dissected by the "Azienda Agricola S. Uberto" accredited slaughtering house (Monterenzio, Bologna) (Provincia di Bologna 2007).

Preparation of faecal samples for spore detection. Faeces were forced out of the rectum and put into a screw cap vial. In the lab, faecal samples were weighted and diluted 10-fold with sterile distilled water. The suspension was decanted for one hour. The precipitate was sifted through a series of metal sieves of decreasing mesh size (800, 400, 150, 60 and 20 μm). Only the material between 150 μm and 20 μm was in size considered for centrifugation (1500 rpm, 3 min) with sterile distilled water because hypogeous fungal spores (and asci) generally range between these dimensions. The supernatant was discharged and the precipitate was treated with 6 modified solutions: 1) NaCl (26.5%); 2) $MgSO_4$ (35%); $ZnSO_4$ (33%); $ZnSO_4$ (70%); 5) sucrose (68%); 6) sucrose gradient (Crede 2007; Gudmundsdottir, Skirnisson 2006; Pet informet 2008; Mitosciences 2007). For the latter, 3 different sucrose solutions (35%, 25% and 15%) were sequentially and carefully layered in a 15 ml tube and, finally, 1 ml of faecal precipitate was layered on top. After sucrose gradient centrifugation (1500 rpm, 2 min), three aliquots (100 μl each) were collected from each 1-ml interval of the gradient, transferred onto a slide and immediately examined.

For the first 5 methods, the faecal precipitate (10 g) was 5-fold diluted with the corresponding solution and centrifuged (1500 rpm, 3min). After centrifugation, new solution was added to the very top of the tube and a cover slip was placed on it for 10-15 min for recovering the floating fungal spores. Cover slips were mounted on a slide and immediately examined under a light microscope. Each sample was treated in triplicate.

Among the six methods tested, the $ZnSO_4$ (70%) solution resulted to be the most reliable to isolate spores and thus it was used for analyzing all faecal samples.

Morphological identification. Fresh samples of fruiting bodies were preliminary identified on the basis of their macroscopic (colour, surface, smell, etc.), and microscopic characteristics (morphological and biometric characteristics of spores and peridium cells) numbered, dried and stored in the herbarium of the Dipartimento di Scienze Agrarie (CMI-Unibo), University of Bologna (Italy). The spores found in the faeces were identified basing on their external characteristics (shape, dimensions and type of ornamentation). TuberKey (Zambonelli et al. 2000) was used as reference for *Tuber* spp. identification whereas Montecchi and Sarasini (2000) monography was used as reference for the species of hypoeous fungi belonging to different genera of ascomycetes and basidiomycetes.

RESULTS

Hypogeous sporoma collection. During the surveys, only a few ascomata of *T. aestivum* and *T. magnatum* were found because the area is regularly visited by other truffle hunters, although truffle harvesting within the Park is forbidden. Other hypogeous Ascomycetes were common in the park, such as *T. excavatum, T. rufum, T. macrosporum T. borchii, T. dryophilum*, *T. brumale*, *Balsamia vulgaris, Stephensia bomycina*, *Genea* spp. Basidiomycetes were only represented by the species of the *Hymenogaster* genus and by *Melanogaster ambiguus* (Tab. 1).

Detection and identification of faecal spores. The data of the 14 wild boars examined and the characteristics of the truffle spores detected in the corresponding faecal samples are shown in Table 2. No fungal spores were detected in 5 animals whereas in 9 ones spores of hypogeous fungi, including *T. magnatum* and *T. aestivum*, were identified (Fig. 1).

Other unidentified fungal spores were present in most faecal contents, including several spores of *Alternaria* spp. (data not shown). Truffle spores were found in both sexes and in the animals from all the Municipalities within the Park.

Table 1
Hypogeous fungi found in "Parco dei Gessi Bolognesi e Calanchi dell'Abbadessa" Regional Park (Bologna, Italy)

Herbarium n.	Species	Date	Municipality
3382	*Balsamia vulgaris* Vittad.	30 12 2006	San Lazzaro
3352		23 01 2007	Ozzano dell'Emilia
3357	*Genea fragrans* (Wallr.) Sacc.	23 01 2007	Ozzano dell'Emilia
2546		15 11 2004	San Lazzaro
3205		08 11 2006	Ozzano dell'Emilia
3375		30 12 2006	San Lazzaro
3372		03 12 2006	San Lazzaro
3351	*Genea lespiaultii* Corda	23 01 2007	Ozzano dell'Emilia
3373	*Genea verrucosa* Vittad.	03 12 2006	San Lazzaro
2466	*Hymenogaster lycoperdineus* Vittad.	27 09 2004	San Lazzaro
3343		24 01 2007	Ozzano dell'Emilia
3353	*Melanogaster ambiguus* (Vittad.) Tul. & C. Tul.	23 01 2007	Ozzano dell'Emilia
3379		30 02 2007	San Lazzaro
3344		24 01 2007	Ozzano dell'Emilia
3867		27 12 2005	San Lazzaro
2552		15 11 2004	San Lazzaro
2550		15 11 2004	San Lazzaro
2452		11 09 2004	Pianoro
1099		25 09 2007	San Lazzaro
2449	*Tuber aestivum* Vittad.	11 09 2004	Pianoro
3192		08 11 2006	Ozzano dell'Emilia
3380		30 12 2006	San Lazzaro
3348	*Tuber borchii* Vittad.	23 01 2007	Ozzano dell'Emilia
3346		23 01 2007	Ozzano dell'Emilia
2544	*Tuber brumale* Vittad.	15 11 2004	S. Lazzaro
3354	*Tuber dryophilum* Tul. & C. Tul.	23 01 2007	Ozzano dell'Emilia
1367		27 09 2004	San Lazzaro
1511	*Tuber excavatum* Vittad.	01 11 2004	Pianoro
2548		15 11 2004	San Lazzaro
3190		08 11 2006	Ozzano dell'Emilia
3197		08 11 2006	Ozzano dell'Emilia
3198		08 11 2006	Ozzano dell'Emilia
3191		08 11 2006	Ozzano dell'Emilia
3189		08 11 2006	Ozzano dell'Emilia
3376		30 12 2006	San Lazzaro
3350		23 01 2007	Ozzano dell'Emilia
3345		23 01 2007	Ozzano dell'Emilia
3185		08 11 2006	Ozzano dell'Emilia
3183	*Tuber macrosporum* Vittad.	08 11 2006	Ozzano dell'Emilia
3184		08 11 2006	Ozzano dell'Emilia
3186		08 11 2006	Ozzano dell'Emilia
3381		30 12 2006	San Lazzaro
3355		23 01 2007	Ozzano dell'Emilia
2547		15 11 2004	San Lazzaro

Herbarium n.	Species	Date	Municipality
3194	*Tuber magnatum* Pico	08 11 2006	Ozzano dell'Emilia
3204		08 11 2006	Ozzano dell'Emilia
3193		08 11 2006	Ozzano dell'Emilia
3374		30 12 2006	San Lazzaro
3241		15 01 2007	Ozzano dell'Emilia
3240		15 01 2007	Ozzano dell'Emilia
3239		15 01 2007	Ozzano dell'Emilia
3242		15 01 2007	Ozzano dell'Emilia
3349		23 01 2007	Ozzano dell'Emilia
3243		15 01 2007	Ozzano dell'Emilia
3255		01 01 2007	Ozzano dell'Emilia
2545		15 11 2004	San Lazzaro
2450	*Tuber rufum* Pico	11 09 2004	Pianoro
2448		11 10 2004	Pianoro
2549		15 11 2004	San Lazzaro
2469		27 09 2004	San Lazzaro
3206		08 11 2006	Ozzano dell'Emilia
3200		08 11 2006	Ozzano dell'Emilia
3195		08 11 2006	Ozzano dell'Emilia
3377		30 12 2006	San Lazzaro
3378		30 12 2006	San Lazzaro
3347		25 01 2007	Ozzano dell'Emilia
1100	*Stephensia bombycina* (Vittad.) Tul.	25 09 2007	San Lazzaro

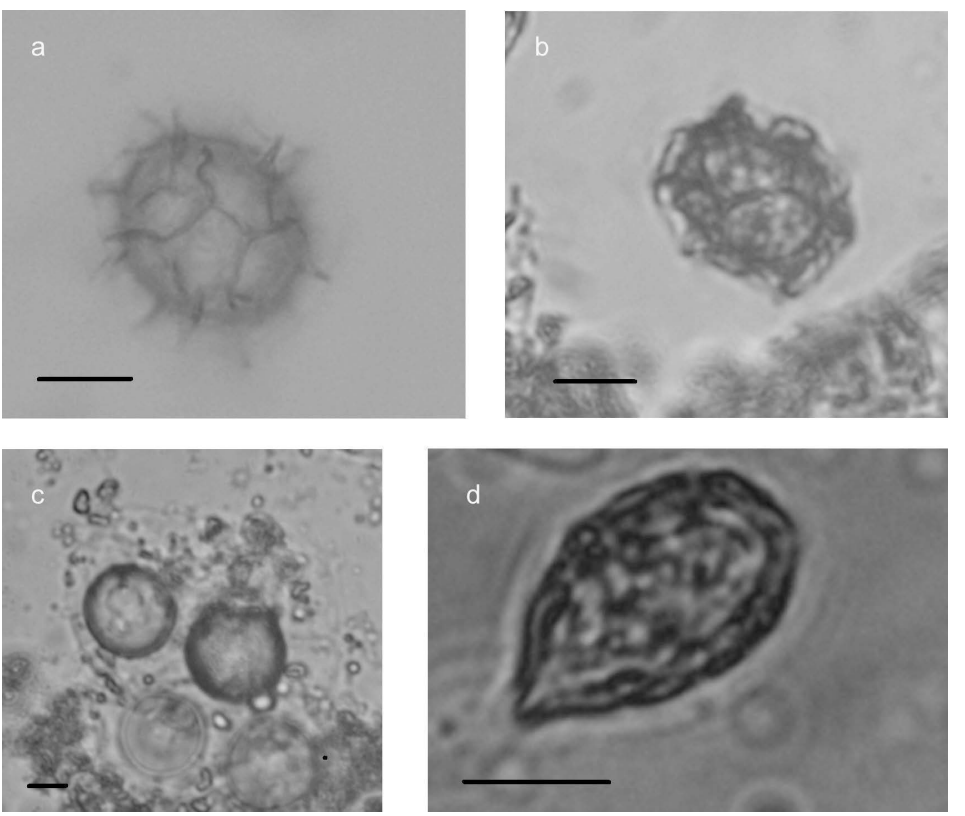

Fig. 1. Spores found in wild boar faeces: a) *Tuber aestivum,* b) *Tuber magnatum,* c) *Stephensia bombycina,* d) *Hymenogaster lycoperdineus.* Scale bars = 10 μm.

Table 2
Wild boar data and characteristics of the spores detected in the faecal material

Animal code	Locality (Municipality)	Sex	Age (months)	Weigth (kg)	Number of spores	Mean dimensions (µm)	Attempt of identification
34532	La Croara (San Lazzaro)	♂	19-22	64	2	20.51 x 23.73	*Tuber magnatum* Pico
34497	Via Gaibola (Bologna)	♂	35	68	<10 6	12.32 x 22.58 21.52 x 22.87	*Hymenogaster lycoperdineus* Vittad. *Stephensia bombycina* (Vittad.) Tul.
34526	Ozzano dell'Emilia	♂	8	23	1 1	15.03 x 11.07 19.20 x 33.69	*Hymenogaster* sp. *Tuber* sp.
34222	Montecalvo (Pianoro)	♀	7	26	1 1	19.61 x 26.32 13.38 x 29.43	*Tuber rufum* Pico *Hymenogaster* sp.
34241	Settefonti (Ozzano dell'Emilia)	♂	7	26			-
34233	Settefonti (Ozzano dell'Emilia)	♀	6	27			-
34530	Settefonti (Ozzano dell'Emilia)	♀	22	65			-
34223	Settefonti (Ozzano dell'Emilia)	♀	25	69	2 4	23.78 x 27.12 13.00 x 21.54	*Genea verrucosa* Vittad. *Hymenogaster lycoperdineus* Vittad.
54321	Acquafredda (Pianoro)	♂	10	32	2	21.40 x 31.03	*Tuber aestivum* Vittad.
34235	Pieve (Ozzano dell'Emilia)	♂	9	26			-
34236	Sabbioni (Ozzano dell'Emilia)	♂	8	26	<10 2	23.55 x 33.41 11.96 x 20.81	*Tuber aestivum* Vittad. *Hymenogaster lycoperdineus* Vittad.
54337	Acquafredda (Pianoro)	♀	9	26	1	20.31 x 32.45	*Tuber* sp.
54389	Ozzano dell'Emilia	♀	7	21			-
54328	Montecalvo (Pianoro)	♀	12	31	3	25.07 x 20.03	*Tuber magnatum* Pico

DISCUSSION

In this study a simple method to detect truffle spores in wild boars' faeces was perfected. This method can be used for ecological studies involving the role of wild animals in hypogeous fungal spore dispersal.

Examining the faeces of just 14 animals hunted in two months, we were able to detect seven hypogeous fungal species including *T. aestivum* and *T. magnatum,* which are the most widespread and economically important species growing in the studied area. These results suggest that the detection of fungal spores in the faeces of wild boars may provide a first rough indication of the presence of hypogeous fungi in an

unknown area, when trained dogs are not available. This methodology may also be useful in countries were truffle harvesting is forbidden even for scientific purposes. For example, in the Czech Republic and in Slovakia truffles are considered endangered species and they are, thus, protected by the law (Grynder et al. 2011).

The spores of hypogeous fungi were found in most of the animals analyzed (9/14) regardless of weight, age or sex. In the past, truffles were harvested with female pigs as it was thought that truffle scent resembled that of the male pig's pheromone (To Tuscany 2012). In fact, the steroid 5α-androst-16-en-3α-ol, which is a major component of the boar pheromone (Claus et al. 1981) has been detected in the black truffle (*Tuber melanosporum).* In the studied area, the lack of preference by female boars for truffles could be explained by the presence of truffle species different from *T. melanosporum*, which probably do not contain this specific pheromone.

Although most animals revealed the presence of hypogeous fungal spores in the faeces, the abundance of spores was poor, limited to just one or few spores in the amount of sample analyzed. This suggests that the wild boar may occasionally eat truffles and that the main damages in natural and cultivated truffières are mainly due to soil disturbance caused by excavation with the snout (Moreno-Arroyo et al. 2005; Salerni et al. 2011; Ricci 2008). On the opposite, other small mammals, like the northern flying squirrels or some marsupials, just eat truffles or simply prefer to eat truffles, whose spores accumulate in faecal pellets (Lehmkuhl et al. 2004; Claridge, Trappe 2005). According to the Claridge and Trappe (2005) classification of mycophagous animals in obligate, preferential, casual, opportunistic or accidental mycophagists, the wild board can be considered an opportunistic mycophagist, ingesting truffles only occasionally, as a seasonal source of food.

In mycophagous animals, there is an evidence of spore germination stimulation by the passage through the digestive system, although the effect on spore metabolic activities may differ among animals and among hypogeous fungal genera (Trappe, Claridge 2005). In fact, most studies on hypogeus asco- and basidiomycetes report a positive effect on spore germination (Colgan, Claridge 2002; Claridge, Trappe 2005). In contrast Miller (1985) reported that the germination of spores of *Tuber* spp. was not stimulated by digestive process in rodents. However, all the studies were carried out only on small hydnophagous mammals or marsupials and never considered large mammals like wild boars (Claridge, Trappe 2005).

Wild boars have larger movements than small sized animals, ranging between 2 and 15 km in one night and up to 300 km for males and 100 km for females during seasonal migrations (Andrzejewski, Jezierski 1978; Singer et al. 1981; Defra 2005). Thus, their role in truffle long distance dispersal may be extremely important if the vitality and infectivity of truffle spores after the passage through the digestive tract, are not negatively affected. In this way, it is possible to speculate that wild boars may have played a key role in postglacial recolonization of truffles from Southern refugia (Murat et al. 2004).

Studies are in progress to verify the effects on vitality and infectivity of truffle spores ingested by a pig, as an animal model, to support these conclusions.

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