ROMA TRE
UNIVERSITÀ DEGLI STUDI
CICLO XXV

SCUOLA DOTTORALE IN BIOLOGIA

SEZIONE **BIODIVERSITÀ ED ANALISI DEGLI ECOSISTEMI**
ANNO ACCEDEMICO 2012-2013

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Co-tutor: prof. **Anna Loy** (Università degli studi del Molise)

Eurasian otter (*Lutra lutra*) in central Italy: non-invasive methods to assess status and conservation of a threatened population

La lontra eurasiatica (*Lutra lutra*) in centro-Italia: metodi non invasivi per accertare lo status e per la conservazione di una popolazione minacciata

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Thesis defense on the 26th February 2013
in front of the following jury:

prof. Bogliani Giuseppe
prof. Camarda Ignazio
prof. Mazzoleni Stefano

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Preface

This thesis is based on two papers, submitted to peer-reviewed journals, derived from data gathered during my PhD research project. It also report new results that will be part of a third peer-reviewed paper to be submitted shortly.

The thesis is structured as follow:

CHAPTER 1. A BRIEF INTRODUCTION to the species with an overview of methodologies used in otter studies. The last part of this chapter presents the Aims of the PhD project.

CHAPTER 2. CAMERA TRAPPING. The chapter is structured around the first manuscript submitted: *"Improving camera traps to increase detection probability of semi-aquatic mammals"*. Further attempts not included into the paper are reported in the last part of the chapter.

CHAPTER 3. NON-INVASIVE GENETICS. The chapter is structured around the second manuscript submitted: *"An optimized procedure to improve genotyping of problematic non-invasive otter (Lutra lutra) samples"*. Final results about spatial structure and relatedness among individuals that were not included into the paper are presented in the last part of the chapter.

CHAPTER 4. DISCUSSION AND CONCLUSIONS. The discussion highlights the importance of the present study for increasing the knowledge on the Eurasian otter in Italy. Conclusions present questions raised from the results of the study, and outline a plan of future perspectives.

CHAPTER 5. REFERENCES.

PAPERS AND CONGRESSES. This session summarizes the two papers submitted, as well as posters and oral communications presented at national or international congresses.

APPENDICES. Report the official Pdf files received by editors of two peer-reviewed journals after papers submission and poster presented to national and international congresses.

Chapter 1. Introduction

The Eurasian otter (*Lutra lutra*)

Generalities

The Eurasian otter *Lutra lutra* (Linnaeus 1758) is a semi-aquatic mustelid belonging to the subfamily Lutrinae. The Eurasian otter has one of the widest distributions among Palaearctic mammals, occurring in Europe, Asia and Africa (Corbet 1966) and includes seven subspecies (Pocock 1941) [FIG. 1]: *L. l. lutra* in Europe and northern Africa; *L. l. nair* in southern India and Sri Lanka; *L. l. monticola* in northern India, Nepal, Bhutan and Myanmar; *L. l. kutab* in northern India–Kashmir; *L. l. aurobrunnea* in Garhwal Himalayas in northern India and higher altitudes in Nepal; *L. l. barang* in southeast Asia (Thailand, Indonesia and Malaysia); and *L. l. chinensis* in southern China and Taiwan (Ruiz Olmo *et al.* 2008).

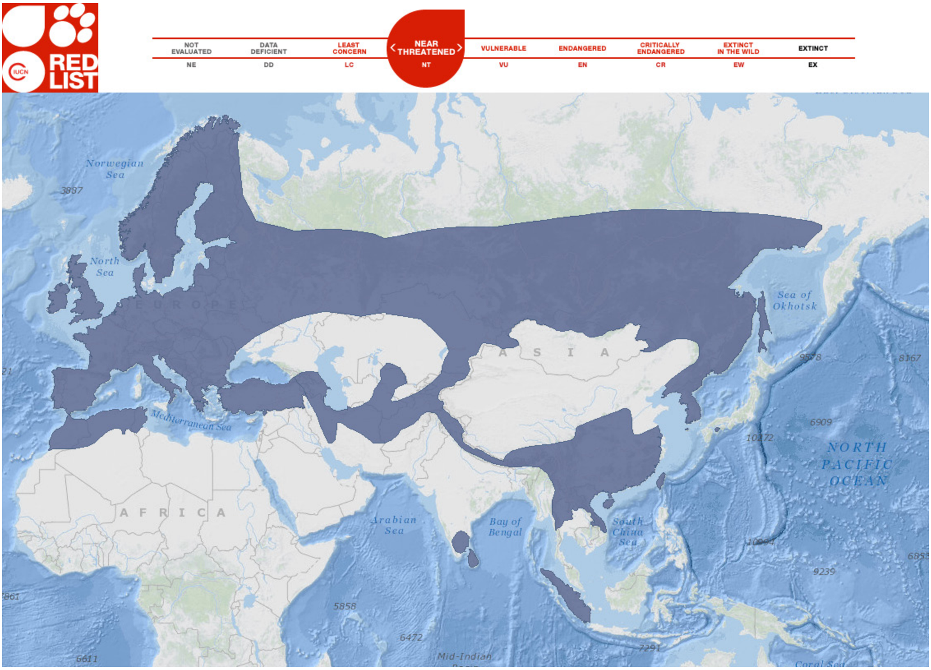
The overall colour is dark brown, slightly lighter underneath and with light patches on the throat. The species is characterized by sexual dimorphism (Moors 1980) with a mean total length (from nose to tail tip) of about 1.0 m for females and 1.2 m for males with an average weight of 7 kg and 10 kg respectively (Kruuk 2006).

HABITAT. The Eurasian otter lives in a wide variety of aquatic habitats, including lakes, rivers, streams, marshes, swamp forests and coastal areas (Mason and Macdonald 1986; Kruuk 2006). In Europe it is found from the sea level up to 1,000 m in the Alps (Ruiz-Olmo and Gosalbez 1997), but in Asia occurs up to 4,120 m in Tibet (Mason and Macdonald 1986).

DIET. Eurasian otters feed mainly on fish (Webb 1975; Ruiz-Olmo and Palazon 1997), but other prey items have been recorded in their diet in variable proportions, *i.e.* insects, reptiles, amphibians, birds, small mammals, and crustaceans (Jenkins and Harper 1980; Adrian and Delibes 1987; Skaren 1993).

BEHAVIOUR. The Eurasian otter is considered mostly solitary although some forms of social groups can be observed (Kruuk and Moorhouse 1991) allowing to grasp the existence of a more complex social system. Cubs remain with the mother for about 10–13 months while males do not care offspring. Male otters have extremely large ranges overlapping with several female territories. Related females may share "group territories" with individual exclusive "core areas" (Kruuk and Moorhouse 1991; Kruuk 2006). A characteristic behaviour commonly used by mammals for intra-specific communication is the scent-marking (Macdonald 1985; Gorman 1990). Data on marking patterns of mustelids are scanty (Hutchings and White 1990) but fundamental for sociobiology understanding. The role of marking has been largely discussed and, according to some authors, scents identify a territory maintained and defended by an individual or a group (Gosling 1982; Gosling and McKay 1990). Most carnivores often mark the territory boundaries but the large and linear-shaped home-ranges of otters (Green *et al.* 1984; Kruuk 1995; Ó Néill *et al.* 2009) make this strategy less advantageous (Hutchings and White 2000). It has been argued that scent-marking in river otters could be associated more with resource presence than with territoriality (Kruuk and Moorhouse 1991; Kruuk 1992). Scents are long-lasting and permit to recognize individuals and their breeding status (Gorman and Trowbridge 1989; Rostain *et al.* 2004), playing a role in intra-specific communication and social interactions.

The two main otter marking signs are scats, also called *spraints*, and *jellies*, produced by anal glands common to all European mustelids (Pocock 1921). Selected marking sites are frequently prominent objects such as stones along river banks or emerging in the middle of the river flow. Marking at the same sites is recurrent and permit to use scent-signs surveys for studying otters.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NORTH PACIFIC OCEAN

Fig. 1 — Range of the Eurasian otter

Conservation status

During the last century, particularly on ‘60s and ‘70s, the species declined throughout Europe, mostly in Central and Western Europe, and experienced a hard habitat reduction and fragmentation all over its distribution range (Foster-Turley *et al.* 1990; Conroy and Chanin 2000). The species became extinct in Luxemburg, Holland, Liechtenstein, Switzerland and in the most of Italy, Spain, France, Germany, Belgium and Great Britain (Ruiz-Olmo *et al.* 2008). In the last decades the species recovered in most European countries and in 2007 was downgraded from Vulnerable (VU) to Near Threatened (NT) category in the IUCN Red List.

In Italy the status of *L. lutra* is still critical. The species disappeared from northern Italy during the ‘80s (Macdonald and Mason 1983a; Cassola 1986) and the decline continued all over the ‘90s [FIG. 2A,B,C], while other European populations were recovering (Conroy and Chanin 2000; Kranz and Toman 2000; Roos *et al.* 2001; Mason and Macdonald 2004). Finally the species went extinct in Central Italy (Reggiani *et al.* 2001a,b; Prigioni *et al.* 2007) and its distribution restricted to the South.

At the beginning of the XXI century an isolated remnant otter population was found in Molise a region of south-central Italy (Loy *et al.* 2004), and signs of recovering were appearing in southern Italy (Prigioni *et al.* 2007). In 2007 signs of a new northward expansion phase in central Italy became evident with the colonization of the river Sangro (De Castro and Loy 2007) [FIG. 2D].

The Italian population is geographically and genetically isolated from other European populations (Randi *et al.* 2003) and it consists at present of two metapopulations: the largest one is located in southern Italy and the smallest one in southern-central Italy (Loy *et al.* 2004; Fusillo *et al.* 2004; Prigioni *et al.* 2007) with an estimated number of individuals of 196–220 and 33–37 respectively (Prigioni *et al.* 2006). The otter was listed as critically endangered (CR) in the national red list (Bulgarini 1998), and a national action plan was recently adopted by the Italian Ministry of Environment (Panzacchi *et al.* 2011).

In the last two years first records of otters were reported in north-east Italy (Righetti 2011; Lapini and Bonesi 2011), following the dispersal of individuals from Austria (Bolzano province) and Slovenia (Friuli). Genetic analyses confirmed that these individuals do not belong to the southern Italian population (Lapini pers. comm.). A small nucleus of otters escaped from captive breeding enclosures is actually present in the Ticino river (north-western Italy). These otters belong to the so-called B-line, *i.e.* individuals derived from a captive population that was crossbred with the Asian subspecies *L. l. barang*. These B-line specimens were declared not suitable for reintroduction in the wild by the IUCN and the reintroduction projects planned by the Regional Parks of Ticino Lombardia and Ticino Piedmont were discouraged by ISPRA (National Institute for the Environmental Protection and Research) (Panzacchi *et al.* 2011).

The species has a high priority for conservation (CITES 1979; Council of Europe 1979; Habitats Directive 92/43/CEE). The Eurasian otter is considered a *flagship* species (Bifolchi and Lodé 2005; Cianfrani *et al.* 2011), a charismatic species that can become a symbol to encourage conservation consciousness (Caro *et al.* 2004; Heywood 1995).

The semi-aquatic habits and the link to freshwater and riparian habitat could also make the otter an umbrella species which offers protection for “background species” (Caro *et al.* 2004; Roberge and Angelstam 2004). Thus, the whole freshwater ecosystems will benefit from otter conservation issues. But successful conservation and management of threatened wildlife require accurate and robust information. The lack of data prevent proper conservation measures so, gaps of knowledge have to be filled up.

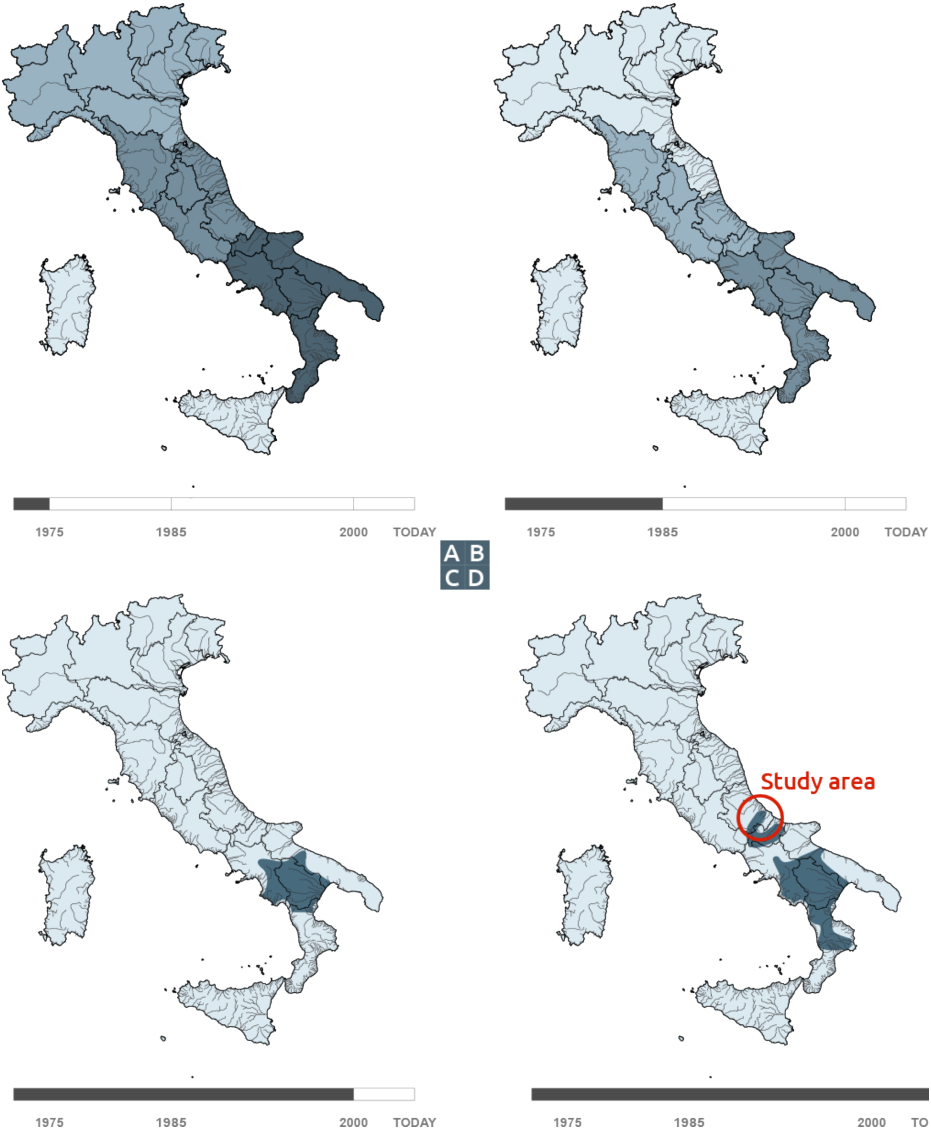
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Fig. 2 — Otter distribution in Italy during the last four decades (darker colors correspond to higher abundance)

Major threats

The major causes of the decline of otter populations in the last century included overharvesting, pollution, habitat destruction and fragmentation (Foster-Turley *et al.* 1990). Whereas harvesting was reduced due to protection acts, many threats still subsist for the species survival in Europe. Chemical pollution concerns several substances such as dieldrin, organochlorines, polychlorinated biphenyls (PCBs) and heavy metals. To date, the most deleterious effects derive from PCBs and heavy metals (Lemarchand *et al.* 2010). PCBs, endocrine disruptive compounds, can alter immunological and nervous systems, beyond causing dermal and ocular alterations. Heavy metals — especially cadmium (Cd), lead (Pb) and mercury (Hg) — can affect several organs and cause reproductive, haematological and immunological deficiencies (Boscher *et al.* 2010). Pollutants affect both individual and population levels.

To date, the most frequent recorded cause of mortality is represented by road kills (Simpson 1997; Hauer *et al.* 2002; Jancke and Giere 2011). Other common causes are entanglements in fish-nets and weakening injuries resulting from intra or inter-specific encounters (Simpson 2006). Some causes of death are probably overestimated due to a bias in the recovered dead individuals. Road killed individuals are more frequently found in respect of otters poached or dead in holts for starvation and other casualties, so giving biased results. Intra-specific fighting are probably more frequent than expected but scars and wounds are discovered only during necropsies (Simpson and Coxon 2000; Simpson 2006).

In Italy, two major threats for otter survival are the habitat destruction and the high instability of freshwater availability over the year, characteristic of Mediterranean areas (Panzacchi *et al.* 2011). Habitat destruction and fragmentation are particularly dangerous for species, such as otters, limited to one or few habitat types (Bright 2000). Both habitat destruction (*e.g.* banks overbuilding, roots and stones removal) and water fluctuations can lead to a hard reduction of food resources. Destruction and alteration of riparian structure also reduce cover and shelter as well as alternative food resources (*e.g.* amphibians and crustaceans). Moreover, the Italian population suffers a serious reduction of genetic variability. This fact, in addition to the reduced abundance, fragmentation of metapopulations and geographical isolation from other European nuclei, could trigger an extinction vortex (Frankman *et al.* 2010; Gilpin and Soulé 1986).

Standard approaches used in wildlife research and in otter studies

Many techniques, often involving handling of animals, can be applied to gather information on species ecology, behaviour and requirements. However, capture programs are risky and often controversial, especially when dealing with rare and endangered species. Radio-tracking is probably the most useful strategy to gather a huge amount of data but involves capture and anaesthesia. In the case of Eurasian otter, it is still more hazardous due to the need of a surgery for implanting intra-peritoneal radio-transmitters (Fernandez-Moran *et al.* 2002). In fact, affix a radio-collar is not possible as the neck is wider than the head and use a radio-backpack may be risky if snagging in underwater branches (Kruuk 2006). To date, in Italy only two wild otters have been radio-tracked (Fusillo 2006) while previously, 6 “line B” otters were experimentally released from the captivity centre of Caramanico Terme and radio-tracked (Mattei *et al.* 2005) along the Aterno-Pescara basin. Due to recent advances in molecular ecology, complementary information to radio-tracking can be gathered by non-invasive genetic sampling.

Distance sampling techniques such as sign surveys, direct observations and counts are common in wildlife research but are mostly unfeasible with elusive and nocturnal species. The historically most used method to study otters is the *spraint* survey. This procedure was standardized in Europe in order to compare data among different periods and sites and it was adopted by the IUCN/SSC Otter Specialist Group (Reuther *et al.* 2000). The area of interest is subdivided in 10×10 km cells and then four sites of freshwater per cell are randomly selected and surveyed. Surveyors cover 600 m of river stretches searching for otter presence signs. Despite the Europe-wide use of *spraint* surveys, an heated discussion arose among researches. The method is used for assessing otter presence and distribution but the evaluation of otter abundance and habitat selection is controversial (Conroy and French 1987; 1991; Jefferies 1986; Kruuk *et al.* 1986; Kruuk and Conroy 1987; Macdonald and Mason 1987; 1991; Ruiz-Olmo *et al.* 2001). Direct observations can be efficiently used only where otters have diurnal habits. At the same time, remote photography and videotaping are widely used in wildlife studies also for nocturnal species.

Due to the high potential of non-invasive genetics to gather information about both individuals, and populations and in order to reduce potentially detrimental impact and disturbance, I decided to use non-invasive methods. On one hand I applied camera trapping to gather information on behaviour at marking sites; on the other hand I coupled the *spraint* survey methodology with non-invasive genetic sampling to assess the status of the otter population living along the river Sangro basin [FIG. 3].

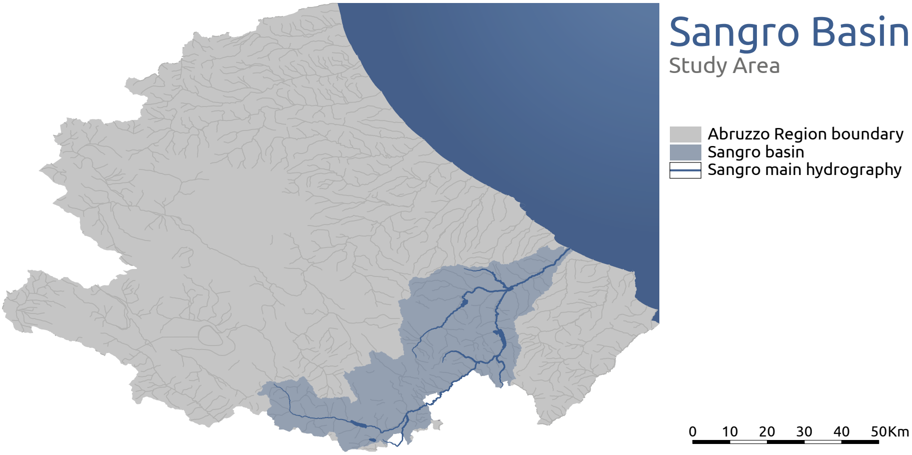

Fig. 3 — Extension of river Sangro basin in Abruzzo region

Aims

For long-term conservation planning we need basic information about ecology, population structure and genetic variability. This kind of data are particularly hard to obtain when dealing with rare and elusive species. The Eurasian otter is still critically endangered in Italy where metapopulations present are highly isolated and fragmented. Moreover, Italian population is genetically isolated and distinguished from other European populations, so representing an Evolutionary Significant Unit (ESU – Moritz 1994; Mucci *et al.* 2010). In the last decade the Eurasian otter is recovering in Italy but no data are available in the new areas of expansion.

Despite its relevant conservation interest, many aspects of the ecology and sociobiology of the Eurasian otter remain unknown (Kruuk, 2006), and this is especially true for the Italian subpopulation.

Studies on Italian otters have been mainly focused on surveys, diet and species distribution modelling (Cianfrani *et al.* 2010; Ottaviani *et al.* 2009; Prigioni *et al.* 2006; Remonti *et al.* 2008; Remonti *et al.* 2010), while very little is known on population structure, and on resource utilization in space and time.

The current study represents the first attempt to obtain detailed data about an otter population living in the river Sangro basin, the northernmost nucleus in Italy. One of the most important challenge in conservation biology are the small and low variable populations where the role of chance prevails and the effects of natural selection are typically reduced (Frankham *et al.* 2005). Moreover the Sangro basin population plays a crucial role for the future expansion of the species northward and so for conservation issues in our Country.

The elusive nature of the species, together with its nocturnal habit and rarity, make difficult to study wild otters and obtain basic data for conservation plans. The overall scope of this thesis was to increase knowledge on otters, and improving non-invasive methods to be most effective in future researches. Major efforts were spent in upgrading camera-trapping devices and genetic analyses protocols in order to overcome the set of problems usually encountered in otter researches.

Chapter 2. Camera trapping

Since the end of XIXth century photography has been used to record wildlife (Guggisberg 1977). In 1890s Georges Shiras developed, for the first time, a system by which wild animals photographed themselves (Kucera and Barrett 2006) and in 1926 Nesbit published the first detailed guide to outdoor photography. Through the 1990s remote photography has been increasingly used in wildlife studies and in 1999 Cutler and Swan reviewed the major previous literature, reporting the main topics investigated: feeding ecology, nesting behaviour and nest predation. Camera trapping now represents a widely used non-invasive method to study wildlife and to answer a variety of questions such as the presence and distribution of rare and endangered species (Surridge *et al.* 1999; Jeganathan *et al.* 2002), behavioural patterns (Bridges *et al.* 2004), comparisons of results with classical sign surveys (Glen and Dickman 2003; Silveira *et al.* 2003; Srbek-Araujo and Chiarello 2005), habitat and activity patterns (Jacamo *et al.* 2004) beyond the capture-recapture models using individual photo identification (Karanth 1995; Marnewick *et al.* 2008).

The Eurasian otter (*Lutra lutra*) can be a suitable target for employing camera traps to answer questions about its biology and behaviour. Nocturnal habit and elusiveness do not permit direct observations, but the characteristic scent marking behaviour at known sites (Kruuk 2006) allows to set remote cameras and record the animals during their nocturnal activities out of the water. However, data on river otters obtained by remote camera trapping are still scanty and many missing data characterize all previous studies (Stevens *et al.* 2004; Garcia de Leaniz *et al.* 2006; Guter *et al.* 2008; Hönigsfeld Adamič and Smole 2011).

During my research project I did test and modified camera traps for otters to increase the record success rate, as described in the following paper (Lerone *et al.*, submitted).

PAPER 1 – Submitted to Wildlife Biology (APPENDIX I)

Improving camera traps to increase detection probability of semi-aquatic mammals

Lerone L., G.M. Carpaneto, A. Loy

ABSTRACT

Camera trapping represents a powerful tool in wildlife research, particularly when dealing with elusive and rare species like the Eurasian otter (*Lutra lutra*). Nevertheless, detection problems arise when trying to detect otters with camera traps at frequent marking sites along rivers. We hypothesized that the temperature difference between the otter emerging from the water and the environment was too low to be detected by the standard infrared sensors (PIR). We designed and tested a new pressure trigger and compared its effectiveness with that of the standard PIR. Results are encouraging, as the new sensor detected after few trapping nights.

KEYWORDS camera trapping, infrared sensor, *Lutra lutra*, otter, semi-aquatic mammals, thermoregulation.

INTRODUCTION

Remote photography in wildlife research increased in the 1990s when commercial digital and low cost cameras became available (Swann *et al.* 2011). Detecting elusive and rare species, recording activity patterns and estimating population parameters are among the challenges of this technology (Karanth and Nichols 1998, Maffei *et al.* 2005, O'Connell *et al.* 2006, Nichols *et al.* 2008). The Eurasian otter (*Lutra lutra*) represents a suitable potential target for camera traps due to its elusiveness, marking behaviour and nocturnal habits. Nevertheless, data on river otters obtained by remote cameras are still very scanty and high levels of missing data characterized most studies (Stevens *et al.* 2004, Garcia de Leaniz *et al.* 2006, Guter *et al.* 2008, Hönigsfeld Adamič and Smole 2011). We hypothesized that detection problem could be due to the thermoregulation system and hair structure (Tarasoff 1974, Khun and Meyer 2010). Using a FLIR ThermaCam B20, Kuhn and Meyer (2009) observed two layers of hairs providing a high thermal insulation and demonstrated that, after swimming bouts, the temperatures of otters surface and water were similar. This high thermal insulation could prevent the standard infrared sensors (PIR) to detect animals temporarily emerging from water whereas few minutes of activity on land are necessary to increase the body temperature (Kuhn and Meyer 2009). Based on these considerations we tested a camera trap provided with a modified trigger specifically designed to overcome this problem and to increase the detection probability of river otters.

METHODS AND RESULTS

Frequent marking sites of river otters were detected during a pilot study for noninvasive genetic sampling of an otter population recently found in the river Sangro (South-Central Italy, Lerone *et al.* 2011). Two passive triggered camera traps (ScoutGuard SG550®) equipped with a PIR sensor were initially set on the riverbanks in correspondence of rocks emerging in the middle of the water course where fresh marking signs (spraints and/or anal jellies) were frequently found. The PIR detects the simultaneous occurrence of movement and temperature changes due to the animal's transit. Highest sensibility was set to record one minute video or three consecutive photos. Cameras were opportunistically checked from June to September 2010 and from June to September 2011. Their correct functioning and setting were tested at each control and confirmed by several other species recorded. After 171 day traps neither videos nor photos of otters were recorded despite the transit of the species was attested by fresh marking signs in at least 16 occasions. To test the hypothesis that body temperature was a critical factor for proper activation of the camera trap, a standard ScoutGuard SG550 was set along a small beach nearby the water course. In this case we expected the otter getting warmer while walking along the bank and becoming visible to the PIR sensor. During 27 day traps one video of an otter walking was recorded, giving us an evidence of the critical role played by body temperature (http://youtu.be/pfj_jA2Gg4Y). One of the two ScoutGuard SG550 was then modified to be activated by a pressure trigger and set together with the non-modified camera as control. This first trial device was made up of two medical tongue depressors [FIG. 4].

We fixed the pressure trigger on marking rocks using a commercial glue and we connected it to the camera by an electric wire passing underwater and constrained with stones. We blinded the PIR of the modified camera to guarantee the solely activation by the pressure trigger. Despite the potential disturbance caused to animals by the device or the smelling glue we did not notice any difference in marking behaviour of otters after camera placement. The coupled cameras were settled during 104 day traps in which we attested otter presence in at least 15 occasions. After only two day traps, the modified camera recorded one video of an otter while the standard one was not activated (http://youtu.be/eJKEL4jkoEs). During the last trapping session some problems arose with rain closing the circuit and thus continuously activating the remote camera. Consequently the memory card was soon filled up and the batteries depleted. To overcome this problem we further improved the device by embedding the pressure trigger on a plastic disk that prevented water to reach the circuits [FIG. 5].

DISCUSSION

Remote passive camera trapping represents a helpful noninvasive alternative method to monitor and investigate wild-

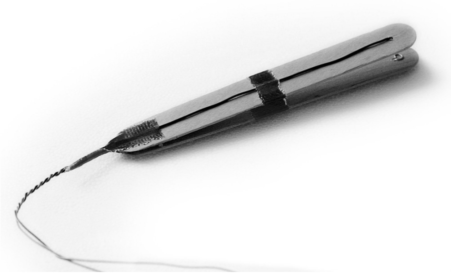

Fig. 4 — Proof-of-concept pressure trigger

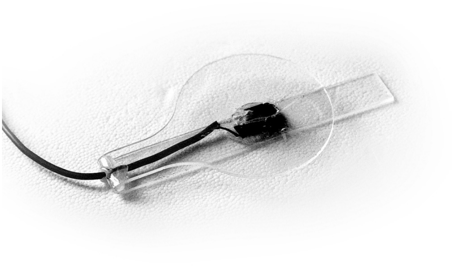

Fig. 5 — Improved pressure trigger provided by E. Centofanti

life presence, abundance, and behaviour (Cutler and Swann 1999, O'Connell *et al.* 2006). This kind of data is particularly difficult to obtain when dealing with shy, rare and endangered species like semi-aquatic mammals. In fact the only accurate data on otters were obtained through continuous videotaping (Guter *et al.* 2008), or by setting infrared beam underwater (Garcia de Leaniz *et al.* 2006). Continuous tape-recording is extremely useful but expensive, as the cameras need to work 24/7, and video post production is very time consuming. By contrast submerged photocells allow to detect otters underwater, but are not suitable to record any terrestrial behaviour and activity. Our pressure trigger modified camera was able to detect otters emerging from water at marking sites with a higher detection probability of a camera trap provided with a standard PIR sensor. According to our preliminary observations the modified camera has a high potential of detecting semi-aquatic mammals that remain undetected to PIR sensors. Further experiments with other semi-aquatic species will likely confirm these previsions and we hope our pressure trigger tested on Eurasian otters could be a first step in such direction.

MANAGEMENT IMPLICATIONS

Semi-aquatic mammals either represent appealing flagship, key and umbrella species for freshwater habitats (Bifolchi and Lodé 2005, Stevens *et al.* 2011), or important issues for the control of alien invasive species (*i.e.* American mink and coypu, Bonesi et al 2004). Gathering data on these elusive species is therefore essential to define population status and design successful management and conservation programs. Moreover, the possibility to observe animal behaviour into the wild could be a fascinating way to approach people to conservation issues and values (Carroll and Meffe 1997). Our pressure trigger modified camera will likely improve the remote data recording of semi-aquatic mammals and gain valuable information for the management programs of these species.

ACKNOWLEDGEMENTS

We thank A. Leone and C. Lerone for their helpfulness during the conception of the first pressure trigger; E. Centofanti (www.fototrappolaggio.com) for designing and providing the improved pressure trigger; L. Quaglietta, A. Argenio, F. Peloso, L. Vielmi, D. Righetti and G. Simonetti for their advices and constant support.

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Further results

During the third year field work, I experimented again the improved pressure trigger of the camera trap (Multipir 12®). During low-moderate raining days the trigger overcame the main problems of circuit closure. A further important implementation consisted to set the trigger with the opening downward with respect to the rock slope. This trick permits a faster slipping of water, that possibly gone into the trigger during plentiful rain.

Moreover, when an upstream dam is opened, the water flow can temporarily submerge the marking stone and activate the camera trap [FIG. 6].

If the dipping time is short, and it occurs during hot days, the trigger dries itself off and the device correctly operates, despite a quicker consumption of batteries and memory card.

In order to overcome problems derived from occasional trigger submersion, to minimize potential disturb at marking sites derived from the smelling glue, and to further

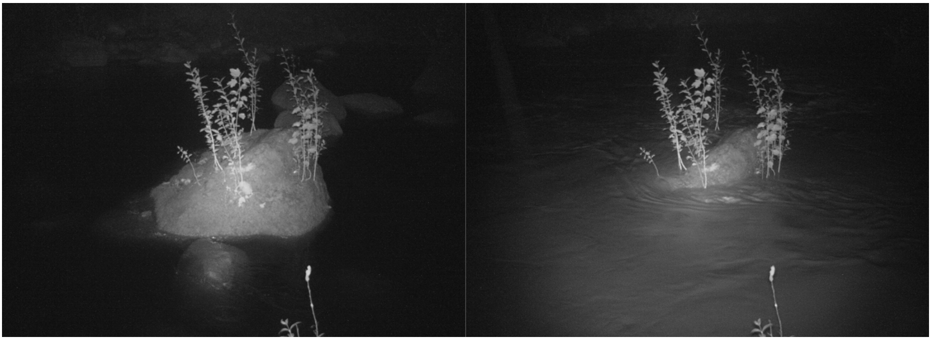

Fig. 6 — The same otter marking site, before (left) and after (right) upstream dam opening

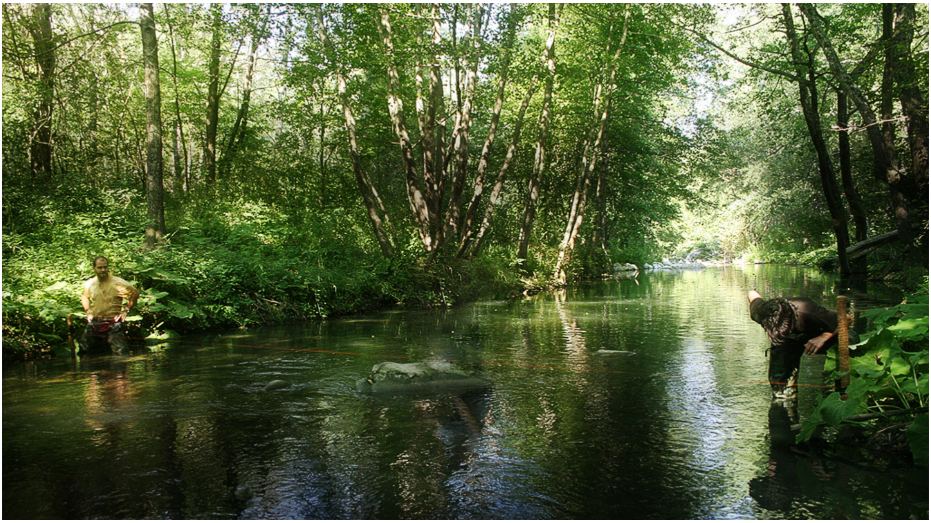

Fig. 7 — Photocell setting (the red beam is simulated)

test the role of body temperature in photo trapping, I added a pair of photocells on the river banks [FIG. 7]. I used a commercial model of photocells for outdoor use, powered with an external 12 V battery. In this case, when the invisible IR beam between photocells was interrupted from an animal transit, the camera started to record or shot.

I started the experiment by setting simultaneously three cameras: a standard one, a camera modified with pressure trigger, and a camera modified by adding activation by photocells. Unfortunately, one of the photocells was stolen shortly after setting and I had to remove all the device in order to prevent the theft of the whole equipment.

Camera traps setting, control and tests are very time-consuming but could give us important information about otters. Despite the small sample size cannot permit any conclusive inference, I could observe that all photos and videos (3 videos and 16 photos) were recorded during nighttime. This datum confirms the mainly nocturnal habits of the recorded otters in our study area.

Moreover, I always spotted single individuals thus suggesting a solitary behaviour. This evidence has to be discussed in the light of the results from the non-invasive genetic sampling that showed evidences of large spatial overlap among individuals (see chapter 4). Therefore, solitary behaviour could be not related to the exclusive use of river stretches, but to exclusive use of some key resources defended by marking behaviour.

A further important datum obtained from camera traps was the detection in the study area of the coypu (*Myocastor coypus*), a South-American rodent introduced in Italy during the last century as furbearing animal.

Even if coypu is known not to directly compete with otters (Pavlov and Kiris 1960) it can represent a serious problem if people do not distinguish between them. Coypu is a problematic invasive alien species and unlike otter, dig underground dens in the river banks and damage cultivated lands for feeding. In both cases it causes economical losses. Mistaken identification could lead to an increase of poaching events against otters. As far as I know the occurrence of coypu and American vison detected during this research project represent the first data of occurrence of these alien species in the river Sangro basin, and will deserve further studies to detect the distribution and problems related to their presence.

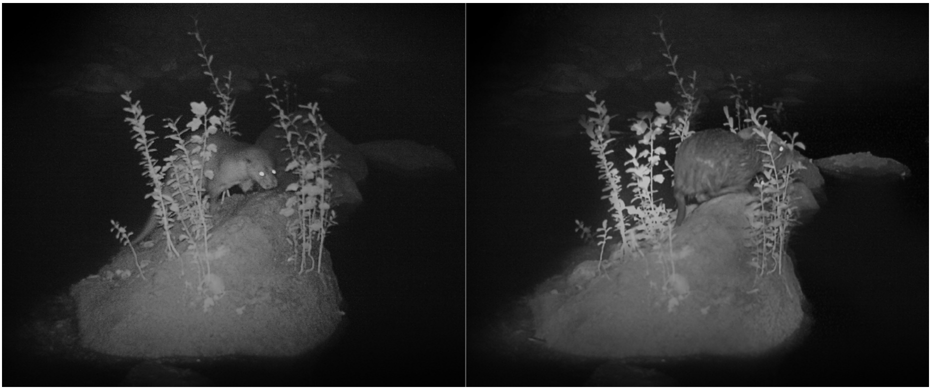

Fig. 8 — *Lutra lutra* and *Myocastor coypus* at the same site in different occasions

Chapter 3. Non-invasive genetics

The modern approach based on ecological genetics focuses on conservation biology topics such as population size and structure, migration and dispersal among populations (gene flow), trade-offs between local selection and genetic drift (Lowe *et al.* 2008). A deep impact in improving abundance and accuracy of this kind of data was provided by the development of PCR-based genetic markers (DeYoung and Honeycutt 2005; Piggott and Taylor 2003). PCR technology permits to amplify DNA starting from a tiny amount of genetic material. This allows to analyse and compare the scanty DNA collected from wildlife in a non-invasive way, *i.e.* from samples like scats, hairs, feathers etc. When dealing with endangered and rare species, the collection of non-invasive samples can be, not only the most valuable, but the solely way to obtain information for conservation and monitoring programs (Kohn and Wayne 1997).

The non-invasive sampling (NGS) was used for the first time in the '90s to collect DNA without disturbing the animals (Höss *et al.* 1992; Morin and Woodruff; Taberlet and Bouvet 1991). To date, many genetic markers exist and can answer to a large amount of biological questions (Kohn and Wayne 1997; Sunnucks 2000; Wan *et al.* 2004). Genetic markers are "simply heritable characters with multiple states„ (Sunnucks 2000) permitting to study and infer processes underlying genetic diversity throughout various hierarchical levels (individuals, demes, populations etc). Nuclear microsatellites are, to date, the most used molecular markers applied in population genetics analyses (Beebee and Rowe 2008). Microsatellites — also called Short Tandem Repeats, STRs — are highly variable tandem repeats of mono to tetra-nucleotides, scattered into the genome (Ellegren 2004). Microsatellite are codominant (both alleles at a locus are visible in heterozygote condition) and display a higher variability when compared with other markers, being highly informative also for intra-population studies. The analysis of several variable microsatellite loci permit to obtain a genetic individual fingerprint that can be used to estimate population abundance and to perform parentage analysis (Blouin 2003; Jones *et al.* 2010; Marucco *et al.* 2011). Microsatellites also allow to assess the genetic status of a population, derived from the analysis of alleles frequencies, heterozygosity and Hardy-Weinberg equilibrium (Goossens *et al.* 2005; Lu *et al.* 2001; Swenson *et al.* 2011) and to make comparisons among populations. Despite the wide range of potential applications of NGS and microsatellites, limitations and risks have to be considered (Piggott and Taylor 2003; Waits and Paetkau 2005). Non-invasive samples, particularly scats, typically present low quality and quantity of target DNA and high amount of degradation agents, PCR inhibitors and non-target genetic material. Moreover, DNA quality can influence the genotyping success and the reliability of results. Two main types of genotyping errors are linked to NGS: allelic dropouts (ADO) when one allele of an heterozygote fails to amplify and false alleles (FA) when the true allele is misgenotyped because of PCR artifacts [FIG. 9].

Errors have to be quantified and incorporated in subsequent analyses in order to refine data accuracy (Buchan *et al.* 2005; Waits and Paetkau 2005; Hájková *et al.* 2009).

The Eurasian otter is a suitable target for application of NGS, being a rare, often nocturnal and secretive species characterized by a recurrent marking behaviour (Hájková *et al.* 2009; Kruuk 2006).

Nevertheless, genetic analyses from otter scats are always problematic, and river otters studies (both *Lutra lutra* and *Lontra canadensis*) displayed lower success rates than other mammal species, only comparable to results obtained for the red wolf (Hájková *et al.* 2009).

Low success rate in otters is likely influenced by the high level of environmental humidity that favours the proliferation of DNA degradation agents as soon as *spraint* deposition occurs (Lampa *et al.* 2008).

My research project specifically devoted many efforts to optimize each step of the NGS protocol, from DNA sampling to sequencing, and to reduce contaminations and potential errors in genotyping results. These efforts are summarized in the protocol described below (Lerone *et al.* submitted — PAPER 2). All the analyses were performed at the genetic laboratory of ISPRA (Ozzano dell'Emilia, BO, Italy), where a high standard of quality is maintained, including the physical separation of extraction, amplification and sequencing rooms. A prior screening step, at three microsatellite loci (OT07, OT14, OT17), was carried out to cut off low quality samples and reduce unreliable genotypes. Low amount of DNA into PCR reaction lead to low accurate results; empirical evidences highlighted that samples which failed at >50% of markers during a screening analysis had ADO rates about 70 times higher than other samples (Paetkau 2003; Waits and Paetkau 2005). So, only samples successfully amplified at 50% or more screening PCRs (3 nuclear microsatellite loci) were analysed at all loci (13 loci). As suggested by previ-

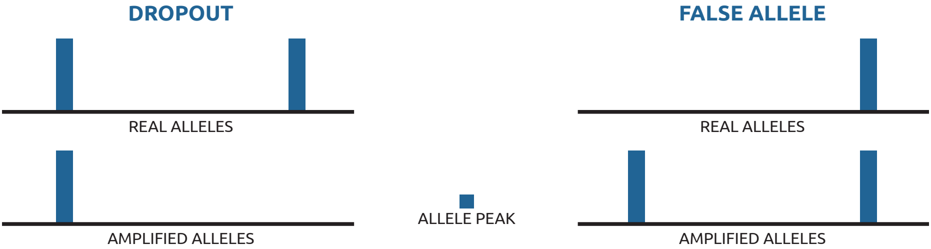

Fig. 9 — Graphic representation of allelic dropout (ADO) and false allele (FA) PCR error types

ous studies (Hájková *et al.* 2009; Taberlet *et al.* 1996), we used a multiple tubes approach (several independent amplification repeats per locus, per sample) all over the analyses, both for screening and for further PCR repeats.

For the first time, we simultaneously used and compared two sets of microsatellite loci (Lut and OT) in order to better characterize and distinguish otter individuals in small, low variable populations.

Results are reported in the paper below.

PAPER 2 – Submitted to Acta Theriologica (APPENDIX II)

An optimized procedure to improve genotyping of problematic non-invasive otter (*Lutra lutra*) samples

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ABSTRACT

Non-invasive genetics is a powerful tool in wildlife research and monitoring, especially when dealing with elusive and rare species like the Eurasian otter (*Lutra lutra*) in Italy. Nevertheless the DNA of otter obtained from scats (spraints) and anal secretions (jellies) appears exposed to very quick degradation processes, and success rate in DNA amplification is much lower than in other carnivores. We collected 191 samples along the river Sangro basin (Italy), recently re-colonized by Eurasian otter. Using two sets of microsatellite loci (Set 1: six Lut loci and Set 2: seven OT loci) we investigated the influence of sample freshness and type on genotyping success. We also tested efficacy of different DNA extraction kits and storage buffer solutions. Finally we compared amplification success rate, allelic dropout (ADO) and false alleles (FA) rates in the two STR loci sets. We obtained a mean amplification success rate of 78.0% and a genotyping success rate of 34.55%. Fresh pure jellies yield the highest amplification success and genotyping rate. The theoretical probability of identity among unrelated individuals and siblings were respectively PID = 0.005 and PID_{sibs} = 0.069 for Set 1, and PID = 0.001 and PID_{sibs} = 0.030 for Set 2. No significant differences in genotyping rates were observed between the two STR sets, but Set 2 loci were more informative for individual identification in our small and low variable population.

KEYWORDS faecal DNA, mammal conservation, microsatellites, molecular scatology, Mustelidae, non-invasive genetic sampling

INTRODUCTION

Successful conservation and management of threatened wildlife requires accurate information on population abundance, structure and genetic variability. This kind of data can be difficult to obtain, especially when dealing with rare and elusive species. Recent technical advances in molecular ecology offer a new accurate tool to monitor wild populations in a non-invasive way (Morin and Woodruff 1996; Kohn and Wayne 1997; Taberlet *et al.* 1999; Piggott and Taylor 2003; DeYoung and Honeycutt 2005).

After a strong decline, in the '80 the Eurasian otter is recovering in many European countries and it has been recently downgraded to the "Near Threatened" category in the IUCN Red List (Ruiz-Olmo *et al.* 2008). In Italy the species is still considered rare and it is listed as "Critically Endangered" in the national red list (Bulgarini et al 1998; Loy *et al.* 2010). The Eurasian otter disappeared from northern Italy during the '80s (Macdonald and Mason 1983a; Cassola 1986, MacDonald and Mason 1994), and its decline continued in central and southern regions all over the '90s, while other European populations were recovering (Conroy and Chanin 2000, Roos et al 2001, Mason and Macdonald 2004, Romanovski 2006). Finally, the otter went extinct in central Italy (Reggiani *et al.* 2001; Prigioni *et al.* 2007), and its distribution was confined to the south with a small geographically and genetically isolated population (Randi *et al.* 2003). The current otter presence in Italy consists of two subpopulations: a larger one in Basilicata (the southernmost) and a smaller one in Abruzzo and Molise (south-central) (Loy *et al.* 2004, 2004, Prigioni *et al.* 2007). Based on the non-invasive genetic sampling (NGS) data available for Italy (Prigioni *et al.* 2006), their abundance was estimated in 196-220 and 33-37 individuals respectively.

According to recent unpublished data, the south-central remnant population is now expanding northward, but the lack of detailed information about its structure and biology represents a serious impediment to the adoption of appropriate management actions and conservation plans. As direct observation are almost unfeasible, non-invasive genetic sampling represents an important opportunity to get precious data on the status and structure of local populations. This is particularly important considering that in Italy the Eurasian otter shows the highest differentiation among the European populations, suggesting a divergence that might reflected local adaptation and ESU (Evolutionary Significant Units) in need of special protection (Randi *et al.* 2003; Mucci *et al.* 2010).

The Eurasian otter (*Lutra lutra*) is a suitable species for applying NGS, due to the typical marking behaviour used for intra-specific communication (Chanin 1985; Gorman and Trowbridge 1989; Kruuk 1992, Kruuk 1995, Ben-David *et al.* 2005). The main challenge concerns the reliability of results due to high levels of genotyping errors (Buchan *et al.* 2005; Waits and Paetkau 2005; Hájková *et al.* 2009). Non-invasive samples usually contain low quality and quantity of target DNA and high amounts of PCR inhibitors and non-target DNA. Genotyping errors have to be quantified and incorporated into subsequent analysis for population studies (Bonin *et al.* 2004; McKelvey and Schwartz 2004, Hájková *et al.* 2006). In this context *L. lutra* is one of the most problematic species, as the DNA in scats is exposed to a very quick degradation process and DNA amplification success rate is usually very low (Jansman *et al.* 2001; Dallas *et al.* 2003; Hung *et al.* 2004; Prigioni *et al.* 2006; Ferrando 2008; Lampa *et al.* 2008; Hájková *et al.* 2009).

Every step from sample collection to DNA amplification presents some critical aspects that can influence genotyping success. Eurasian river otters use two main types of scent marking signs for communication: spraints and anal jellies (Ben-David *et al.* 2005; Kruuk 2006). Spraints consist most of food remains while jellies are slimy secretion produced by characteristic anal glands. Both spraints and jellies can be used for genetic analysis with different amplification success (Fike *et al.* 2004; Hájková *et al.* 2006; Lampa *et al.* 2008; Hájková *et al.* 2009). Our aim is to test and compare different types of non-invasive samples, extraction kits, storage buffer solutions and two sets of nuclear microsatellite loci in order to establish the most successful storage method and the best working loci for genotyping wild otters.

MATERIALS AND METHODS

Sample collection and storage

Sampling season extended from May to September 2011. Spraints and anal jellies were collected along the river Sangro and its main tributaries in southern-central Italy, at the northern boundary of the small subpopulation range. Marking sites were detected during a pilot study (from May to October 2010, Lerone *et al.* 2011) through linear sampling transects conducted according to the European standard method (Reuther *et al.* 2000). Daily checks at marking sites were carried out to guarantee the collection of very fresh samples, within 24 hours from deposition. We also collected samples of medium freshness at less responsive marking sites. Samples were identified as spraint, jelly or mixed spraint, containing jelly in addition to prey remains (Hájková *et al.* 2009), immediately stored in ethanol 96% in 1.5 ml tubes and preserved at -20°C until DNA extraction. A subset of 30 samples were stored both in ethanol 96% and in WCLB (White Cell Lysis Buffer) in order to test for the effect of conservation buffer solution on PCR amplification success.

DNA extraction

The time spent between sample collection and DNA extraction represents one of the main factors that influences PCR amplification success (Frantzen *et al.* 1998; Murphy *et al.* 2007; Lampa *et al.* 2008). Storage time ranged from 3 to 24 days and we compared genotyping success of two categories of samples: extracted within and after six days from collection. As Lampa *et al.* (2008) reported a significantly difference in amplification success rate between two different extraction methods (Chelex® 100 and QIAmp® DNA Stool Mini Kit), DNA from 13 non-invasive samples was extracted according to the manufacturer protocols of two different kits, DNeasy Blood and Tissue Kit (Qiagen) and ZR Genomic DNA II Kit (Zymo Research). The successive samples were only extracted with the Qiagen Kit. Negative controls were included in each extraction to monitor contamination.

DNA amplification and sequencing

We analysed otter samples with two sets of nuclear microsatellite loci. In particular, we characterized six loci “Lut” (Set 1: Lut453, Lut604, Lut701, Lut832, Lut833, Lut902; Dallas *et al.*, 1998) already used in other studies (Dallas *et al.* 2002; Kalz *et al.* 2006; Hájková *et al.* 2009) and seven loci, “OT” (Set 2: OT04, OT05, OT07, OT14, OT17, OT19, OT22; Huang *et al.* 2005) so far used only once for *L. lutra* in Europe by Koelewijn *et al.* 2010). All samples were initially screened for three microsatellite loci (OT07, OT14, OT17) with four independent repeats per locus. Due to the high cost of analysis and the low quality of non-invasive samples, only samples with 50% or more positive screening PCR (Peatkau 2003) were analysed at all microsatellite loci and with ZFX/ZFY sequences for molecular sexing (Mucci *et al.* 2007). To overcome the main sources of genotyping errors (false allele, allelic dropout and sporadic contamination), a multiple tubes approach (Taberlet *et al.* 1996) was used both for initial screening and the following analyses. Negative controls were always included in the analysis to monitor contamination. PCRs were performed in 8 µl volumes containing 2 µl of DNA. In order to optimize costs and time spent for analyses, we run five multiplex PCR (M1 included Lut453, OT05, OT22; M2 included Lut833, OT19; M3 included Lut604, Lut 701; M4 included Lut832, Lut902; M5 included OT17, OT07) and one simplex PCR for OT04. When further amplifications were needed to verify data, only simplex PCR were used. Amplifications were performed using the following thermal profile: a first denaturating step at 94°C for 2 min; 45 cycles at 94°C for 40 s, 55°C for 60 s and 72°C for 90 s; and a final extension step at 72°C for 10 min. PCR products were electrophoresed with GeneScantm – 350 ROX (Applied Biosystem) as marker ladder in an ABI 3130XL sequencer and allele sizes were scored using the software GENEMAPPER v.4.0® (Applied Biosystems). False alleles and allelic dropouts were estimated using GIMLET software® (Valière 2002) and allele frequencies and unique genotypes identified by GenAlEx 6® (Peakall and Smouse 2006).

RESULTS

Sample collection and storage

A total of 191 samples were collected, including spraints, jellies and mixed spraints. As stated above, 30 samples were stored in two different storage buffer solutions, *i.e.* ethanol 96% and WCLB. A percentage of 46.7 (n=14) of the samples stored in ethanol 96% were successfully extracted and amplified, while only 20.0% (n=6) samples stored in WCLB gave reliable genotypes. Results of Pearson with Yates' correction test (X^2=3.675, P=0.055) indicated that ethanol 96% represents a better buffer solution for non-invasive samples. Since the difference is poorly significant, likely due to the low sample size (n=20), this result will need further confirmation. No significant differences in PCR success rate emerged among samples stored in different buffers (Wilcoxon test V=0.074, p=0.074).

We were able to record data about freshness for 182 of the 191 samples. We categorized as "very fresh" the samples collected within 24 hours from deposition (n=108), "fresh" samples that appeared moist despite the uncertainty of deposition time (n=44) and "medium/old" samples which appeared dry (n=30). We obtained significant differences among samples (Pearson X^2=8.66; p=0.01) with very fresh samples providing the highest genotyping success rate (38.0%) followed by fresh (27.3%) and medium/old (13.3%) samples. Despite a difference was observed in the total genotyping success, no significant differences arose in positive PCR rate among very fresh, fresh and medium/old samples (Kruskal-Wallis test: H=1.22, df=2, P=0.54).

DNA extraction

To test the effects of storage time on genotyping success we divided our samples in two groups, *i.e.* extracted within six days (n=61) and extracted after six days (n=72) from collection. The rates of success were 37.7% and 33.3% respectively, with no significant difference (Pearson $X^2=0.118$, $p=0.73$). Extraction methods can also influence genotyping success (Lampa *et al.* 2008). Despite genotyping success for the samples extracted with Qiagen Kit and Zymo Research kit were 53.84% and 15.38% respectively, statistical test did not show a significant difference between the two kits (Pearson $X^2=2.719$, $p=0.10$), likely due to the limited sample size (n=13).

DNA amplification and sequencing

Sample type

We completely genotyped 34.55% of the total samples but when considering separately the different types of samples, significant differences arose. The highest genotyping success was obtained from pure jellies (68.8%), followed by mixed spraints (28.0%), and spraints (25.0%) (Kruskal-Wallis test: $H=11.089$, $p=0.003$). Analysing the two main PCR error types, allelic dropout (ADO) and false alleles (FA), we obtained a significant difference in the ADO rate but not in FA rate (Kruskal-Wallis test: ADO $H=14.22$, $p<0.001$; FA $H=0.162$, $p=0.92$). A post-hoc pairwise comparisons indicated high significant differences in positive PCR ($W=255$, $p<0.001$) and ADO ($W=201$, $p<0.001$) rates between jellies and spraints and significant differences in ADO rate between jellies and mixed spraints ($W=34$, $p<0.05$) and between mixed spraints and spraints ($W=253$, $p<0.001$).

Different microsatellite loci sets

The mean values of PCR success rate, ADO and FA rates are shown in [TAB. 1]. All loci but one (Lut453) were polymorphic [TAB. 1] and allele number ranged

	PCR+	ADO	FA	Na	Ne	Ho	He
LUT701	0.64	0.276	0.006	3	2.649	0.429	0.622
LUT453	0.84	0.163	0.007	1	1.000	0.000	0.000
LUT604	0.92	0.267	0.000	2	1.849	0.714	0.459
LUT832	0.76	0.226	0.003	2	1.960	0.429	0.490
LUT833	0.84	0.219	0.020	3	2.074	0.857	0.518
LUT902	0.84	0.230	0.007	2	1.690	0.571	0.408
OT04	0.73	0.262	0.000	2	1.912	0.643	0.477
OT05	0.77	0.217	0.005	2	1.600	0.357	0.375
OT07	0.72	0.202	0.005	2	1.690	0.429	0.408
OT14	0.89	0.148	0.003	2	1.600	0.357	0.375
OT17	0.74	0.266	0.009	3	2.178	0.714	0.541
OT19	0.62	0.238	0.000	4	2.126	0.538	0.530
OT22	0.87	0.247	0.008	3	2.240	0.786	0.554

Tab. 1 — Positive PCR rates (PCR+); allelic drop out rates (ADO) and false allele rates (FA); number of alleles (Na); n effective allele (Ne); observed (Ho) and expected (He) heterozigosity from one to four. We found no significant differences between results from the two sets of loci. The main percentage values of positive PCR were respectively 81% and 76% (t=0.8331, p=0.42) while the ADO rates were 0.23 and 0.22 (t=0.1964, p=0.85) and the FA rates 0.05 and 0.04 (t=0.9287, p=0.38). Nevertheless the OT loci resulted more variable than Lut loci. We identified 18 alleles in OT loci *vs* 13 alleles in Lut loci [TAB. 2]. This variability allowed to better characterize individuals in our small population. As the allele size may influence the amplification success and ADO rates (Buchan *et al.* 2005; Broquet *et al.* 2007) we analysed the effect of the mean molecular weight of DNA fragments on PCR success rate and ADO. No relationship was found between fragment weight and allelic dropout rate (p=0.16) while median allele size influenced PCR success rate (r2=0.78, p=3.3E-05). The best fit line is showed in [FIG. 10].

	Allele	Allele frequencies
LUT701	**202**	**0.286**
	206	**0.500**
	210	**0.214**
LUT453	**125**	**1.000**
LUT604	**129**	**0.643**
	131	**0.357**
LUT832	**188**	**0.571**
	196	**0.429**
LUT833	**147**	**0.571**
	155	**0.393**
	163	**0.036**
LUT902	**147**	**0.714**
	151	**0.286**
OT04	**176**	**0.607**
	204	**0.393**
OT05	**175**	**0.750**
	179	**0.250**
OT07	**200**	**0.714**
	204	**0.286**
OT14	**120**	**0.750**
	124	**0.250**
OT17	**145**	**0.071**
	153	**0.571**
	157	**0.357**
OT19	**211**	**0.077**
	215	**0.154**
	223	**0.115**
	227	**0.654**
OT22	**148**	**0.536**
	152	**0.393**
	164	**0.071**

Tab. 2 — Microsatellite loci, alleles and allele frequencies

We also analysed the theoretical probability of identity among unrelated (PID) or sibling individuals (PID_{sibs}) [FIG. 11].

The critical value of PI was 0.001 (six loci) while PI_{sibs} was 0.002 (13 loci). The characterization of six loci (Lut701, OT19, OT22, OT 17, Lut 833 and Lut 832) resulted adequate in absence of siblings while the entire panel of 13 microsatellite loci was necessary to distinguish individuals if siblings were present.

All loci resulted in Hardy-Weinberg equilibrium (p<0.001) and only six on a total of 78 comparisons between loci resulted in linkage disequilibrium.

Results confirmed the presence of at least 14 otters in the sampling area. Molecular sexing was carried out according to the protocol of Mucci and Randi (2007). False allele errors for ZFX/ZFY sequences did not occur while we experimented a mean ADO rate of 0.337 and 77% of positive PCRs.

DISCUSSION

The genotyping success rate on non-invasive samples in our study was 34.55%, a value falling within the range of prior studies on river otters (Dallas *et al.* 2003; Hung *et al.* 2004; Kalz *et al.* 2006; Prigioni *et al.* 2006; Arrendal *et al.* 2007; Ferrando *et al.* 2008; Lanszki *et al.* 2008; Hájková *et al.* 2009; Mowry *et al.* 2011). Jellies collected within the 24 h from deposition had the highest genotyping success rate (68.8%), consistently with previous reported results (Coxon *et al.* 1999; Lampa *et al.* 2008; Hájková *et al.* 2009; Mowry *et al.* 2011). We estimated the rates of the main genotyping errors, allelic drop out and false alleles in order to avoid unreliable results (Kalz *et al.* 2006; Hájková *et al.* 2009;

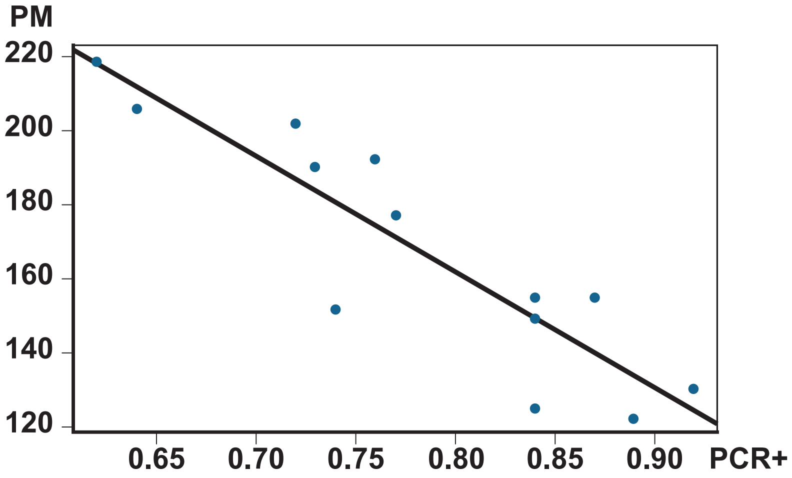

Fig. 10 — The best fit line between PCR success rates and DNA fragment sizes

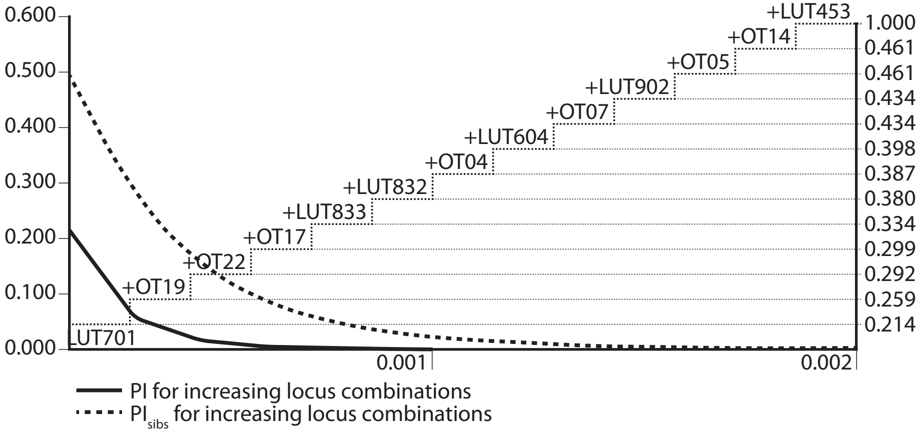

Fig. 11 — Theoretical expected probability of identity values based on allele frequencies (PI and PI$_{sibs}$). The PI values by locus are reported on the right

Spiering *et al.*; 2009, Bjŏrklund 2010; Guertin *et al.* 2012). The mean frequencies of ADO and FA in our study were 22.8% and 0.6% respectively, with allelic dropout rate higher than that found in other studies (Ferrando *et al.*; 2008; Hájková *et al.* 2009) but lower than the value obtained by Lampa *et al.* (2008). Despite the extraction methods could influence the genotyping success (Lampa *et al.* 2008), we did not found significant differences between extractions performed with Zymo Research Kit and Qiagen Kit, even if our results could have been influenced by the small sample size. Slightly differences in genotyping success were found between the two storage buffer solutions, ethanol 96% and WCLB but also in this case the sample size should be increased. We compared two sets of microsatellite loci and neither the percentage of amplification success nor the rates in allelic drop outs and false alleles differed between the two sets in a significant way. Nevertheless the characterization of the OT loci (Huang *et al.* 2005) allowed a better identification of individual genotypes in our small and isolated otter population, owning a very low genetic variability. The OT microsatellite loci could even better work in more variable populations. According with Buchan *et al.* (2005) we detected an influence of DNA fragment size on genotyping success. On the contrary we did not observe any increase in the ADO rate.

Direct observation or radio-tracking of rare and elusive species is difficult and expensive, but the need for demographic and genetic data is more urgent than ever, especially for small populations. The traditional spraint survey method used to estimate the abundance in Eurasian otter populations is being quickly improved by the addition of non-invasive genetic data. Non-invasive genetics provides more reliable data, but there is the need to increase the success rate and the reliability of information that can be obtained from this approach. Our results confirmed that it is important to collect, where possible, fresh pure jellies that guarantee higher rates of positive PCR and lower genotyping error rates. Also, the usage of the OT microsatellite loci developed by Huang *et al.* (2005), combined with the standard Lut loci could represent a promising option to increase the reliability of individual recognition and the characterization of small and low variable populations, the most urgent target of conservation biology.

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Non-invasive Genetic Sampling of otters in the Sangro river Basin (Abruzzo, Italy). Preliminary results

Further improvement attempts

DNA extraction and amplification protocols are described in the above submitted paper (PAPER 2) but here we list the various attempts to increase the amount of target DNA and analysis success. We initially attempted to amplify the whole genome found in each sample by WGA (Whole Genome Amplification). We used the REPLI-g Mini Qiagen®, according to manufacturer protocol. DNA fragments in the reaction mix compete for nucleotides and Taq DNA polymerase that is randomly fastened to the genetic material present in the sample. No results were obtained, likely due to the solely amplification of prey DNA, as the most of *spraints* contained more prey's than otter's DNA. Following these negative outputs we dismissed this costly and time-consuming procedure and devoted more efforts to field work to increase the number of fresh scat (*spraint*) samples.

Genotypes were initially obtained by GeneMapper v. 4.0® (Applied Biosystems). Matches were checked using GIMLET software® (Valière 2002). Where genotypes matched at all but one or two alleles, we review pherograms and, in case, performed additional four PCR repeats at uncertain loci.

In order to increase the non-invasive samples of target DNA quality and quantity, I also built and set of four hair-traps [FIG. 12]. Traps were wooden boxes with several springs stretched, disposed in a manner that permit their snap at the animal transit, so collecting hair. After five weeks of hair traps setting, I found 4 hair samples. Hair samples were observed by an optical microscope, allowing to distinguish hairs from three mustelids (*Lutra lutra*, *Martes foina*, and *Neovison vison*) from other species. To distinguish among these species, DNA was extracted from hair bulbs with the DNeasy Blood and Tissue Kit® (Qiagen) according to manufacturer protocol. Two mitochondrial DNA regions were sequenced: d-loop (non-coding region involved in the replication and expression of mitochondrial genome) and cytochrome b (coding region). MtDNA is commonly used to recognize species due to its characteristic high quantity in a single cell (copies range 10-2500). MtDNA, in particular cytochrome-b gene (*cytb*), is characterized by just a moderate intraspecific var-

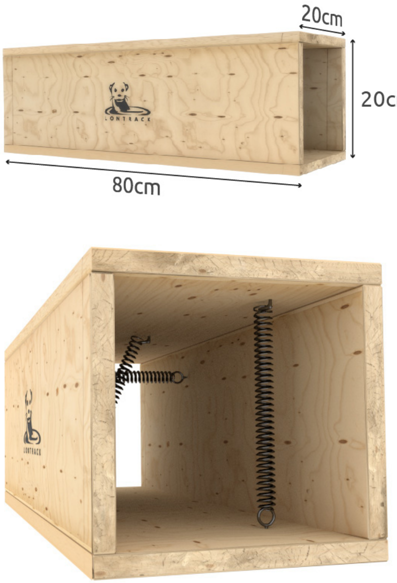

Fig. 12 — Wooden hair trap

iation so resulting very useful for species identification (Kohn and Wayne 1997). MtDNA sequencing confirmed that the hair trapped belong to the American vison, *Neovison vison* ($p<10E-17$). The presence of a fur farming nearby could explain this recovery. The American vison is an allochthonous species that share with Eurasian otter semi-aquatic habits and habitat, however being more generalist and opportunistic. The abundance of American visons in the area needs further investigations such as the potentially detrimental overlap with otters. Fur traps could be a powerful tool for improving DNA quality but is a very time consuming method. Animals need to naturalize with traps and controls have to be performed frequently to prevent contamination and degradation. So, and due to the uncertainty of results, I decided to abandon the attempt that in any case could be worthwhile to try again with the Eurasian otter.

Results

SPRAINTS AND JELLIES

From April 2011 to October 2012 (two sampling seasons: April-September 2011 and June-September 2012) a total of 235 samples were collected from 62 marking sites found along 116 km of the main course and tributaries of the river Sangro [FIG. 13].

The mean genotyping success was 31.5% (samples 2011-2012) and the most successful samples were very fresh *jellies*, *i.e.* anal gland secretions used as marking signs. Unfortunately, as reported in other studies (Hájková *et al.* 2009), *jellies* were rarer than *spraints* (47 *jellies*; 160 *spraint*; 28 mixed *spraints i.e. jelly* mixed to prey remains), and no differences arose between males and females in marking type deposited (Pearson $X^2=2.92$).

INDIVIDUAL GENOTYPES

Genotypes were initially obtained by GeneMapper v. 4.0® (Applied Biosystems). Matches were checked using GIMLET software® (Valière 2002). Where genotypes matched at all but one or two alleles, we review pherograms and, in case, performed additional four PCR repeats at uncertain loci.

Genotyping of all scats and *jellies* at 13 microsatellite loci allowed to assess the pres-

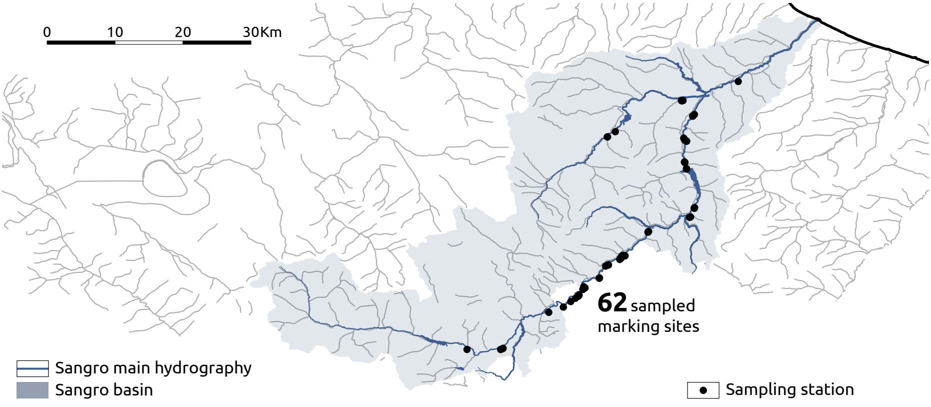

Fig. 13 — 62 Sampling stations along rivers Sangro and Aventino

ence in the river of at least 14 otters, 4 females, 8 males and 2 individuals of uncertain molecular sexing. The last two individuals (F5 and F6) were considered females due to the solely amplification of the female allele. Nevertheless, a small uncertainty remained and we decided to test these individual both as females and males in parentage analyses. All but three genotypes (F5, F6, M6) were confirmed by at least 2 recapture events (*i.e.* successful genotyping of samples collected at different marking sites or dates).

Among the 14 genotypes we identified two individuals, one male (M2) and one female (F2) with identical genotypes at the 13 analysed microsatellite loci. To verify the result we reanalysed samples for both the 13 microsatellite loci and for ZFX/ZFY sequences. Although molecular sexing confirmed the presence of two different individuals, we attempted to distinguish them by analysing four additional microsatellite loci: Lut715, Lut733, Lut782 and Lut818. Unfortunately, all of them resulted monomorphic. Due to molecular sexing results, recaptures (12 for F2 and 8 for M2) and coherence with spatial data we are confident, despite the low Probability of Identity value (P_{sibs}=0.002) reported in the PAPER 2 that these otters share the same genotype (at 17 microsatellite loci) but are distinct individuals.

SPATIAL ANALYSIS AND MEAN DENSITY

The geographical coordinates were recorded (Garmin® GPSmap 60CSx) at each sampling site, permitting to map the ascertain location of individuals. The nature of the data, cannot permit to define the real individual home-ranges but allow to assess the minimum distances covered in a given time period and the overlapping among individuals. Recaptures permit to discover sites repeatedly used by one or more otters and to follow individuals in the course of time. The assessed genotypes give us the opportunity to check for relatedness among individuals and make hypotheses about social

Individual	Recapture	Time span	Max distance covered
F1	2	02/05/2011 – 08/05/2011	25 m
F2	12	03/06/2011 – 07/08/2012	11,850 m
F3	4	28/07/2011 – 15/05/2011	4,500 m
F4	2	28/04/2011 – 21/08/2011	5,550 m
F5	1	22/07/2011	-
F6	1	24/09/2011	-
M1	2	03/06/2011 – 05/08/2011	650 m
M2	8	03/05/2011 – 16/09/2011	29,300 m
M3	23	05/04/2011 – 27/08/2012	40,500 m
M4	4	15/09/2011 – 23/09/2011	29,250 m
M5	2	07/08/2012 – 08/08/2011	-
M6	1	14/07/2012	-
M7	3	08/08/2012	14,650 m
M8	2	18/09/2012 – 21/09/2012	25,950 m

Tab. 3 — Individual otter information

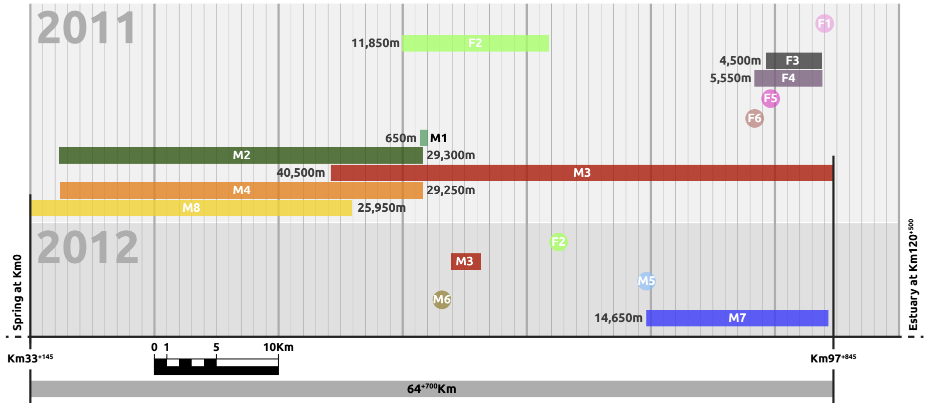

Fig. 14 — Different colour bars and circles represent distinct individuals spatially distributed along the river Sangro. Circle symbolize individuals captured only once. There are also reported the distances between the first upstream and the last downstream samples collected and river spring and estuary respectively

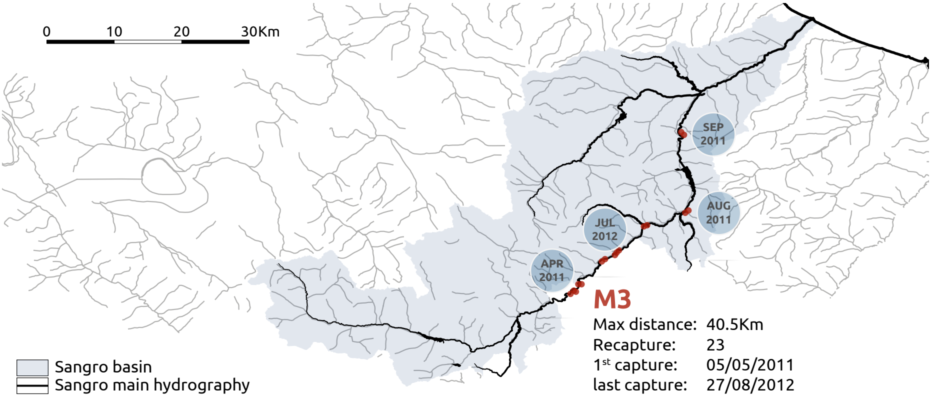
0 10 20 30Km
SEP 2011
AUG 2011
JUL 2012
APR 2011
M3
Max distance: 40.5Km
Recapture: 23
1st capture: 05/05/2011
last capture: 27/08/2012
Sangro basin
Sangro main hydrography

Fig. 15 — Maximum span covered by a single individual

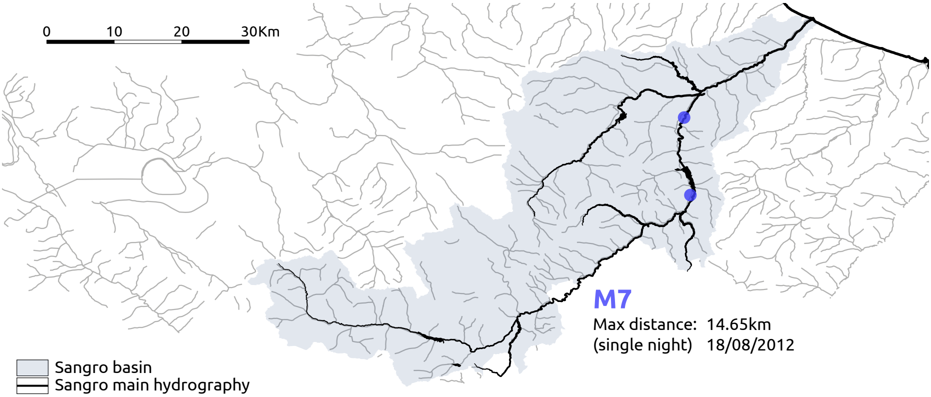
0 10 20 30Km
M7
Max distance: 14.65km
(single night) 18/08/2012
Sangro basin
Sangro main hydrography

Fig. 16 — Maximum distance covered during a single night

structure and spatial distribution. In [TAB. 3] are reported for each individual: the total number of recaptures, the time period from the first to the last recapture and the minimum distance covered during this time lapse.

Of the 14 otters, 9 were captured only during 2011 (F1, F3, F4, F5, F6, M1, M2, M4, M8), 2 were captured both during 2011 and 2012 (F2 and M3) and 3 were captured only during 2012 (M5, M6, M7). To estimate the population density, we considered the 11 otters sampled during the first field season, assuming their presence during the period April-October 2011, supported by no evidence of changes in marking sites (on the contrary, several new marking sites were recorded where new genotypes were sampled during 2012). We calculated density using the total river stretch distance among samples (64.700 km) and we obtained a mean density of 0.17 otters per km.

We analysed the spatial overlapping among individuals in order to infer the spatial structure of the otter population. [FIG. 14] is a graphic representation of distances covered by each individuals along river Sangro and the subsequent overlappings among otters during the two sampling seasons.

The maximum distance was 40.500 km, covered by the male M3 which overlapped with 4 males and 6 females during 2011. In [FIG. 15] is reported the spatial location of M3. The DNA of M3 was repeatedly re-collected along its marking area without presenting a directionality of movements. M3 did not act as a dispersing otter but marked continuously in both directions. Even if M3 covered at least 8.860 km in a single night (02/08/2011), the largest distance recorded, covered during a single night, was 14.650 km by M7 (08/08/12) as reported in [FIG. 16].

[FIG. 14] shows several, both inter and intra-sexual spatial overlapping. For individuals with a longer capture history, we could infer not only a broad temporal overlapping with other otters but in some cases we recorded the presence of more individuals during the same night in the same sampling site or at few meters apart. This is the case of F2-M1 (03/05/11 and 05/08/11); F2-M3 (03/06/11 and 02/08/11); M1-M3 (05/08/11); F3-F4 (28/04/11); M3-M4 (23/09/11). In order to make more informative hypotheses about the spatial structure of the population we analysed genotypes to infer potential relatedness among individuals.

Parentage analysis

Kinship relationships among individuals are fundamental to understand many aspects of spatial distribution, mating behavioural and ecological features valuable in conservation biology. Nevertheless, pedigrees are rarely known when studying wild populations (Wang 2004). Several methods can be used to infer relatedness (Blouin 2003) and nuclear microsatellites are suitable genetic markers for parentage analysis (Jones *et al.* 2010). To infer relatedness among individuals, we used the overall sample (13 individuals) and not only 2011 samples, since it did not matter if individuals were dead or alive for assessing parentage.

After checking for Hardy-Weinberg equilibrium and linkage disequilibrium, assumptions for using the software, we used PARENTE software package (Cercueil *et al.* 2002) to assess maternal and paternal probabilities. We had no information about parental or offspring identities, so we input all individuals as potential parents and offspring. In [TAB. 4] and [TAB. 5] were reported respectively, maternal and paternal probabilities.

I is the number of incompatibilities between potential offspring and parents (*i.e.* number of loci for which the candidate offspring and parents have no common alleles) while P is the probability of the candidate parents being the true parents. Independently of P values, we only accepted 1 as maximum incompatibility and then we only considered parental probability >50%.

PARENTE, using a pairwise approach, compares pairs of individual genotypes (dyad) and calculate a parentage probability. PARENTE also returns the best candidate mother/father pair for each individual, results (P≥50% and maximum 1 incompatibility selected) are presented in [TAB. 6].

To obtain further information we then used COLONY software (Jones and Wang 2009), to identify potential full-sib families. The software permits to input allele frequencies and error rates to be included into the analyses. COLONY, using a group approach (the simultaneous analysis and comparison of the entire sample) highlighted the presence of five full-sib families [TAB. 7]. Each row represents a potential full-sib family. The inclusive probability, Prob(Inc.), is the probability that all individuals listed on the family row are FullSibs (*i.e.* share both parents). Prob(Exc.), the exclusive probability, is the probability that all individuals of the full-sib family and no other individuals are

Offspring	Mother	I	P
F2	F4	0	0.998
F4	F1	0	0.545
M1	F2	0	0.607
M2	F2	0	0.967
M5	F2	0	0.992
M7	F2	0	0.994
M8	F6	1	0.518
F5	F4	0	0.778
F6	F1	0	0.884

Tab. 4 — Maternal probabilities

Offspring	Father	I	P
F1	F6?	0	0.544
F2	M2	0	0.664
M2	M1	0	0.538
M3	M4	0	0.662
M4	M3	0	0.663
M5	M7	0	0.999
M6	M7	1	0.532
M7	M5	0	0.999
F5	F6?	0	0.857
F6	F5?	0	0.936

Tab. 5 — Paternal probabilities (F5? and F6? due to sex uncertainty)

Offspring	Mother	Father	I	P
F1	F4	M2	0	0.66
M2	F1	M1	0	0.50
M5	F1	M7	0	0.99
M7	F1	M5	1	0.99
F5	F4	F6	1	0.76
F6	F1	F5	1	0.83

Tab. 6 — Paternal pair probabilities

FullSibs with this family. [TAB. 7] shows that F3 constitutes a group by itself so being not related to any other otters sampled. The other inferred four groups incorporates two or more individuals. A clearer representation of FullSibs is presented in [FIG. 17] where there are also reported inferred HalfSibs. As highlighted by coloured lines, F3 is the solely otter unrelated with others. An important aspect to underline is that, when no data are available about certain parent-offspring relationships, it is difficult for softwares to distinguish between parent-offspring (PO) and FullSibship (FS) relationships. PO and FS dyads share the same relatedness coefficient (r) measuring the dyad overall identity by descendant (IBD). This is likely the reason for which we can infer, for the same dyad, a PO relationship using PARENTE and a FS relationship using COLONY.

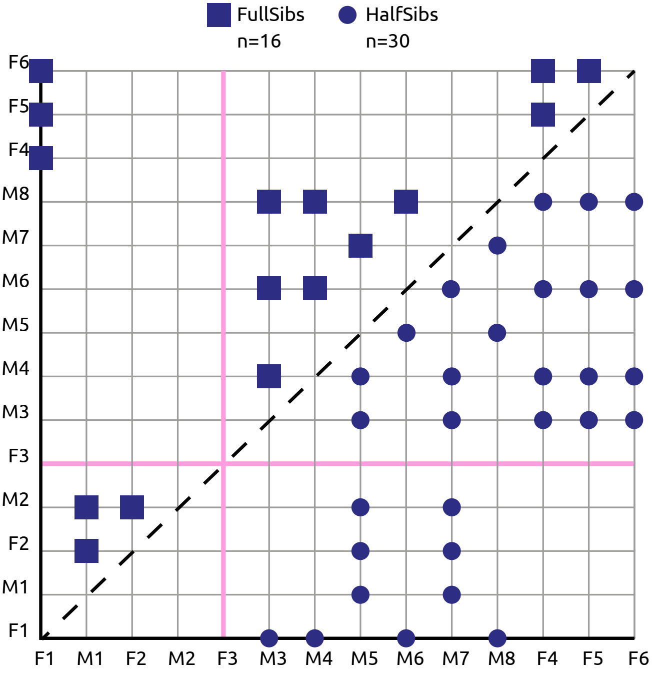

Fig. 17 — Full and HalfSibs inferred relationships among individuals

FullSibs Family	Prob(Inc.)	Prob(Exc.)	Member 1	Member 2	Member 3	Member 4
1	0.1573	0.1572	F1	F4	F5	F6
2	0.9994	0.9665	M1	F2	M2	
3	1.0000	1.0000	F3			
4	0.9734	0.9734	M3	M4	M6	M8
5	0.9396	0.9396	M5	M7		

Tab. 7 — Five potential Fullsibs families inferred by COLONY

Chapter 4. Discussion and conclusions

Discussion

Non-invasive genetic sampling combined with standard survey, camera and hair trapping allowed to gather important information on the biology and ecology of the small and isolated population of Eurasian otters (*L. lutra*) living in the Sangro river basin.

Specifically, our results gave the first insights into the spatial and temporal interactions of Italian otters. In fact, the only non-invasive genetic study conducted in Italy, was able to estimate population abundance in the core area of the species but did not investigate social interactions (Prigioni *et al.* 2006), while the two wild otters radio-tracked in 2004-2005 were living in two separate river courses, thus no data were available about their spatial or temporal interactions. (Fusillo 2006).

Otter distribution and range dynamic in the Sangro basin

Spraint surveys remain one of the most valuable method to monitor otter distribution in a quick and replicable way (Reuther *et al.* 2000). During the third field season (May-October 2012), the whole Sangro basin was checked by two surveyors, following the standard otter survey protocol recommended by IUCN/SSC Otter Specialist Group, in order to give the actual picture of otter occurrence [FIG. 18]. On a total number of 82 linear transects we obtained 24 positive (29%) and 58 (71%) negatives sites. Due to the recognisability of otter marking signs, no doubts arose about presences. On the other hand, at least some of the recorded absences could be false negatives due to imperfect detectability (MacKenzie *et al.* 2006; Reuther *et al.* 2000). Especially when searching for elusive species, is very common for animals to be undetected during presence/absence surveys. Presence/absence data, together with variables recorded during repeated surveys, will permit to monitor otter distribution and to estimate occupancy in order to address some fundamental topics such as specie-habitat relationship and metapopulation dynamics. The 2012 otter survey was the first step, after the re-colonization of the Sangro basin, to systematically assess the species distribution to be compared with past and future data. The Sangro river was previously surveyed by Mason and Macdonald on 1982 and Febbo and Pellegrini during the period 1982-1985 reporting zero positive sites. During 2000-2002 and 2006-2007 inspections were recorded respectively one and nine positive sites (De Castro and Loy 2007; Loy *et al.* 2004). Data comparison permitted to highlight the Sangro basin re-colonization by otters during the last decade while previous connectivity models (Loy *et al.* 2009) allowed to infer that the dispersion of the species likely occurred from the neighbour Volturno basin (Molise). Future NGS analyses extended to the river basins of Molise will likely confirm this hypothesis.

Otter density, spatial and temporal overlap

By combining genetic and spatial data of 2011 we obtained a mean otter density of 0.17 otters/km of watercourse. This value considers only the river stretches that gave positive genotyping results. Due to otter movements, it could be included a buffer zone of 5 km up and downstream from the outer successfully genotyped marking sites. Considering the enlarged area, we obtained a density of 0.15 otters/km. In both cases, our results are consistent with previous reported densities. Prigioni *et al.* (2006) estimated, by non-invasive genetics, a mean density of 0.18–0.20 otters/km in Southern Italy (Pollino National Park – Calabria and Basilicata regions) where it is likely present the largest Italian metapopulation. Estimated Eurasian otter densities range from 0.012 otters/km in England (Kruuk *et al.* 1993) to 1.14 otters/km in Germany (Ansorge 1994; Hauer *et al.* 2002). Most previous studies estimating otter densities were based on *spraint* surveys, but advances in molecular ecology are providing a more accurate tool to infer population abundance.

Spatial and NGS data recorded at each marking site gave us a snap-shot of the population structure along river Sangro. Results gave us evidences of large spatial and temporal overlap among individuals, suggesting a more flexible social system than reported in the literature. Previous studies agree in that male otters have extremely large ranges overlapping with smaller ranges of several females, while related females share “group territories” with individual exclusive “core areas” (Kruuk and Moorhouse 1991; Hung *et al.* 2004; Kruuk 2006; Quaglietta *et al.* 2011).

When accounting for individual relatedness derived from our data, a more defined social structure emerged. All related individual otters detected in the study area overlapped and shared at least part of their ranges. Related females (F1, F4, F5, F6) shared the downstream sampled area [FIG. 19]. This observed female overlap matches with the hypothesis of related female groups (Kruuk and Moorhouse 1991, Kruuk 2006). An unexpected result was the wide overlap observed among related males, particularly M1–M2 and M3–M4–M8 during 2011, and the couples M5–M7 and M3–M6 during 2012 [FullSibs in Tab. 7]. As non-invasive genetic analyses do not give information about individual age we could not assess if all overlapping males were adults or sub-adults. Looking at capture histories, we cannot exclude that M1, M4 and M8 were dispersing juvenile males with one-way movements from their natal area, while the large range of M3 suggests that it was likely an adult resident. Previous findings did not record any extensive overlap among adult males, thus our results could represent the first evidence of “males related groups” not previously recorded.

The lack of females in the upstream sampled area could be due to the failure in genotyping their *spraints*.

It is important to remind the low genotyping success rate characteristic of non-invasive otter *spraints* (Hájková 2009, Lerone et al, submitted). Although in a completely different scenario (Shetlands), Kruuk (2002) observed that males apparently *spraint*ed more than females during summer. If a seasonality was present, a bias in the sampling could have been caused by the female low marking rate.

The classical mustelid socio-biology predicts resource-based female home-ranges (Johnson *et al.* 2000), then, another potential explanation for female absence in the upper watercourse could be the shortage of food resources. Unfortunately we did not investigate this aspect which should deserve further attention.

The number of recaptures can hardly influence estimates of individual ranges so we cannot compare all individual in our population. Nevertheless, for three individuals (F2, M2, M3) we have comparable capture events distributed over several months to infer about their ranges of activity. F2 continuously marked at least 11.850 km while M2 and M3 scent-marked at least along 29.300 and 40.500 km respectively. Data fall into the home-range estimates recorded in previous studies: 12–30 km for females and 21–67 km for males (Durbin 1996; Green *et al.* 1984; Ruiz-Olmo *et al.* 1995; Saavedra 2002).

In Italy, radio-tracking data are available for only two wild otters (female home-range 30 km; male home-range 36 km; Fusillo 20006) and for six Line-B otters (females mean home-range 29 km; males mean home-range 44 km; Mattei *et al.* 2005). Our data do not largely differ from previous reported estimates but a larger sample size is needed to confirm home-range size in the Sangro basin.

Hunting and scent marking behaviour

Despite remote camera trapping is helpful to monitor and investigate wildlife presence, abundance, and behaviour (Cutler & Swann 1999, O'Connell *et al.* 2006), this kind of data are difficult to obtain when dealing with shy and rare species such as otters and other semi-aquatic mammals. Camera trapping sensors improved during this research project allowed to increase success rate of video trapping (Lerone *et al.* submitted).

During the present research, videos and photos recorded the nocturnal habits of otters in the study area, in accordance with most previous studies (Garcia de Leaniz *et al.* 2006; Kruuk 2006; Mason and Macdonald 1986). In contrast with the high spatial overlap among individuals recorded through NGS, camera traps also recorded a solitary behaviour of otters in the Sangro basin, apparently confirming the solitary nature. of the Eurasian otters (Erlidge 1967, 1968; Kruuk 1995, 2006; Macdonald and Mason 1983b).

We obtained DNA from *spraints* and *jellies*, used by otters to scent-mark. Although scent-marking is fundamental for social communication, little is known about marking patterns of most mustelid species (Hutchings *et al.* 2000). As many other carnivores, mustelids often concentrate marks close to territory boundaries. Nevertheless, where resources are more scantly distributed, such as in riverine habitats, and when individuals have large linear ranges, such as otters, it could be more effective to defend key resources instead of the whole territory (Hutchings *et al.* 2000, Kruuk 1992). Another hypothesis is that, if familiar groups or some grade of social organization exist, scent-marking may have a main role in communication within individuals sharing the same areas (Kruuk 2006).

Since extensive variations in social organization, habits and spatial distribution have been recorded for several mustelids among different study areas, seasons and years (Carter and Rosas 1997; Hornocker *et al.* 1983; Johnson *et al.* 2000; Reid *et al.* 1994; Sandell 1986), we are aware that other data are needed from different parts of the range to ultimately describe the biology of the Italian otters. Nevertheless our combined approach gave a first important contribution to the comprehension of range dynamics, population density, social structure and use of resources by otters in Italy.

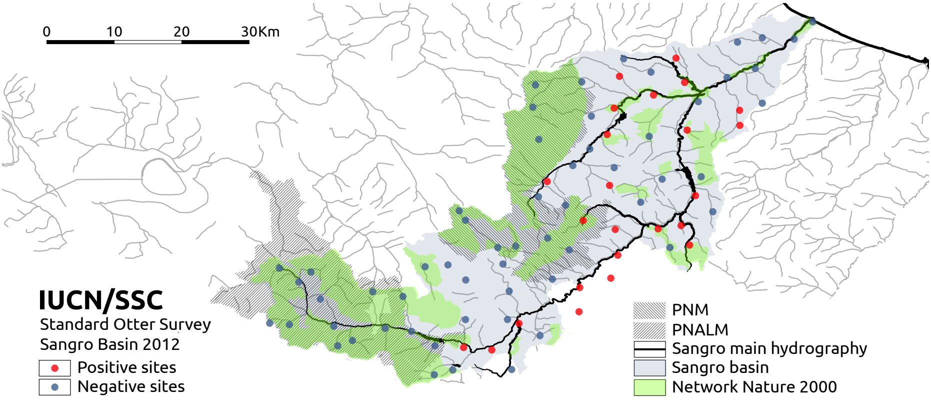
0
10
20
30Km
IUCN/SSC
Standard Otter Survey
Sangro Basin 2012
● Positive sites
● Negative sites
▨ PNM
▨ PNALM
Sangro main hydrography
Sangro basin
🟩 Network Nature 2000

Fig. 18 — IUCN/SSC Standard Otter Survey on Sangro basin in 2012

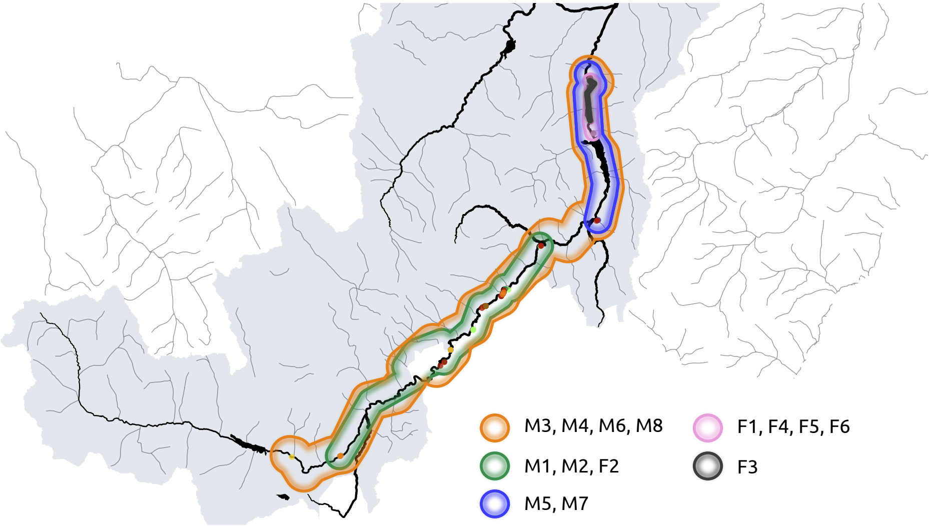
M3, M4, M6, M8
M1, M2, F2
M5, M7
F1, F4, F5, F6
F3

Fig. 19 — Familiar clusters' spatial distribution

Conclusions

The Eurasian otter (*Lutra lutra*) has a high priority for conservation in Europe (CITES 1979; Council of Europe 1979; Habitats Directive 92/43/CEE) and especially in Italy, where it is still listed as Critically Endangered in the national red list (Bulgarini 1998). Despite the high risk of extinction, data on the biology and ecology of the Italian otter population are scanty and fragmented.

Combining otter standard survey, camera trapping and non-invasive genetic sampling provided new insights on the range dynamic, density, scent-marking behaviour, spatial interactions, and social structure of otters in a new colonized area, *i.e.* the Sangro river basin, located at the boundary of the otter Italian range.

Comparison with previous surveys revealed a very active dynamic of the otter range in the last ten years. This was witnessed by the rapid colonization of most of the main water stretches of the river basin.

The survey also accounted for the first otter record in the Majella National Park, but raised some concerns on the potentialities of further expansion in specific parts of the river basin, especially the National Park of Abruzzo, Lazio and Molise, where the Barrea dam likely acts as a barrier preventing animals from expanding upstream. This is in contrast with most data on otters in Europe, where dams are not considered to limit otter dispersal. The barrier effect is probably related to the specific design of the Barrea dam, being embedded within a steep rock canyon. Further studies are needed to assess permeability to otters of this area to allow the occupation of suitable vacant river stretches in the National Parks of Abruzzo Lazio and Molise and Majella, where the otter can receive special protection and act as a flagship species for freshwater bodies conservation.

Non-invasive genetics, particularly microsatellite analysis, permitted to obtain a genetic fingerprint of 14 individuals and to account for major population genetic features that will be essential for future conservation planning of this population.

Specifically, we ascertained the low genetic variability of the population living in the river Sangro. For this reason, it is of primary importance to favour dispersal and genetic flow by preserving and improving the connectivity among neighbouring river basins.

To distinguish among individuals we had to sequence up to 17 microsatellite loci. This stresses the need of new specific and more polymorphic microsatellite loci in order to better characterize small and low variable populations, the most urgent target of conservation biology.

The results from NGS allowed to identify at least 14 otters, 8 males and 6 females, living in the main course and tributaries of the river basin. A large spatial overlap was observed among both males and females belonging to the same full-sibs family cluster. This aspect has to be further investigated because may suggest a more complex social system than reported by other studies in Europe.

Despite NGS highlighted a broad spatial and temporal overlap, camera trapping attested the solitary and nocturnal habits of otters in the study area. These results could support the hypothesis of marking behaviour as tool to communicate resource patch exploitation in a small temporal scale, rather than a way to defence rich resources patches or exclusive territories, as previously stated.

The camera and hair trapping also detected the presence of two alien species, *Myocastor coypus* and *Neovison vison* in the study area. Despite these species are known to have little impact on otters, their potential detrimental effects on the freshwater ecosystem and biodiversity of the Sangro river basin deserves future attention.

Otters, as flagship species, could provide protection for the whole riverine ecosystems and the Sangro basin could play a strategic role for the species conservation in our country. It is the northernmost basin occupied by otters within the smallest nucleus in south-central Italy. The Sangro is the largest river basin of the northern nucleus, and it could likely be able to support a viable population of otters, thus it represents a key area for the future expansion of the otter northward. It also includes a well structured network of Natura 2000 sites and two National Parks which could guarantee a high degree of protection. As a consequence, strong efforts should be devote in the future to favour the further occupation of river stretches still vacant within the basin, specifically the upper and lower portions. This objective could be achieved through the preservation of pristine riparian vegetation belts, restoration of riparian vegetation in unsuitable areas (*i.e.* the Zittola tributary), and to mitigate the barrier effect of the Barrea dam. The camera and hair trapping allowed to detect the presence of two alien species, *Myocastor coypus* and *Neovison vison*. The potential detrimental effects of these species on Eurasian otter needs be further investigated and eventually mitigated.

Rarely we have the opportunity to directly observe natural re-colonisations and non-invasive genetics give us the chance to follow individuals in time and space and to disclose basic but little known aspects such as social and spatial structure and dispersal patterns.

The Eurasian otter is an appealing species, suitable to become a symbol for the conservation of freshwater habitats and biodiversity. Otters, as flagship species, can provide protection for the whole riverine ecosystems, we need data to provide protection for otters.

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Papers

Lerone L., G.M. Carpaneto, A. Loy. *Improving camera traps to increase detection probability of semi-aquatic mammals*. Submitted to Wildlife Biology (APPENDIX I)

Lerone L. C. Mengoni, A. Loy, G.M. Carpaneto, E. Randi. *An optimized procedure to improve genotyping of problematic non-invasive otter (Lutra lutra) samples*. Submitted to Acta Theriologica (APPENDIX II)

Congresses

Lerone L., G.M. Carpaneto, A. Loy. *Why camera traps fail to record otter presence*. IUCN-XIth International Otter Colloquium – Otters in a warming world (Pavia, Italy, 2011). *Hystrix – The Italian Journal of Mammalogy* (n.s.), Suppl. 2011: 32. (APPENDIX III)

Lerone L., Mengoni C., Randi E., Carpaneto G. M., Loy A., 2011. *Non-invasive genetic sampling of Eurasian otter in its Italian northern range*. IUCN-XIth International Otter Colloquium – Otters in a warming world (Pavia, Italy, 2011). *Hystrix – The Italian Journal of Mammalogy* (n.s.), Suppl. 2011: 109. (APPENDIX IV)

Lerone L., Carpaneto G.M., Loy A., 2012. *Improvements to camera trapping applied in wild otter research studies. Hystrix – The Italian Journal of Mammalogy* (n.s.) in press (APPENDIX V)

Lerone L., Mengoni C., Randi E., Carpaneto G.M., Loy A., 2012. *Genetic structure and individual identification of eurasian otter (Lutra lutra) population along river Sangro basin. Hystrix – The Italian Journal of Mammalogy* (n.s.) in press (APPENDIX VI)

Future events

Lerone L., Mengoni C., Randi E., Carpaneto G. M., Loy A., 2013. *An improved non-invasive genetic sampling protocol for otters. First insights into a peripheral Italian population*. Poster presentation, in list for a speed oral presentation. *Musteloid conference*, University of Oxford 18th–21st March 2013.

Wildlife Biology

Improving camera traps to increase detection probability of semi-aquatic mammals

--Manuscript Draft--

Manuscript Number:	WILDBIO-D-12-00102
Full Title:	Improving camera traps to increase detection probability of semi-aquatic mammals
Short Title:	
Article Type:	Short Communications
Keywords:	camera trapping; infrared sensor; Lutra lutra; otter; semi-aquatic mammal; thermoregulation
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Abstract:	Camera trapping represents a powerful tool in wildlife research, particularly when dealing with elusive and rare species like the Eurasian otter (Lutra lutra). Nevertheless, detection problems arise when trying to detect otters with camera traps at frequent marking sites along rivers. We hypothesized that the temperature difference between the otter emerging from the water and the environment was too low to be detected by the standard infrared sensors (PIR). We designed and tested a new pressure trigger and compared its effectiveness with that of the standard PIR. Results are encouraging, as the new sensor detected after few trapping nights.

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Acta Theriologica

An optimized procedure to improve genotyping of problematic non-invasive otter (Lutra lutra) samples

--Manuscript Draft--

Manuscript Number:	ACTH-D-12-00152
Full Title:	An optimized procedure to improve genotyping of problematic non-invasive otter (Lutra lutra) samples
Article Type:	Original Paper
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Abstract:	Non-invasive genetics is a powerful tool in wildlife research and monitoring, especially when dealing with elusive and rare species like the Eurasian otter (Lutra lutra) in Italy. Nevertheless the DNA of otter obtained from scats (spraints) and anal secretions (jellies) appears exposed to very quick degradation processes, and success rate in DNA amplification is much lower than in other carnivores. We collected 191 samples along the river Sangro basin (Italy), recently re-colonized by Eurasian otter. Using two sets of microsatellite loci (Set 1: six Lut loci and Set 2: seven OT loci) we investigated the influence of sample freshness and type on genotyping success. We also tested efficacy of different DNA extraction kits and storage buffer solutions. Finally we compared amplification success rate, allelic dropout (ADO) and false alleles (FA) rates in the two STR loci sets. We obtained a mean amplification success rate of 78.0% and a genotyping success rate of 34.55%. Fresh pure jellies yield the highest amplification success and genotyping rate. The theoretical probability of identity among unrelated individuals and siblings were respectively PID = 0.005 and PIDsibs = 0.069 for Set1, and PID = 0.001 and PIDsibs = 0.030 for Set2. No significant differences in genotyping rates were observed between the two STR sets, but Set 2 loci were more informative for individual identification in our small and low variable population.
Suggested Reviewers:	Cino Pertoldi cino@ebd.csic.es Experienced researcher on conservation genetics applied to European otter.

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IUCN - XIth International Otter Colloquium - Otters in a warming world - Pavia, Italy, 2011

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WHY CAMERA TRAPS FAIL TO RECORD OTTER PRESENCE

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Abruzzo
L'Aquila
River Sangro

Introduction

Camera trapping, also called remote photography, turned into a useful method in wildlife research in the early 1990s, when commercial camera traps became available (Swann *et al.*, 2010). Detection of rare and elusive species, as well as observation of natural behavior, are among its applications. Compared with classic sampling methods, such as trapping, radio tracking and direct observation, photo and video trapping have the advantage of being lesser invasive despite they provide accurate data. Low invasiveness is particularly important when studying endangered species and when we would not interfere with natural behavior of wild animals. To date, there are few data on camera trapping used in river otter's studies (Stevens *et al.*, 2004; Kuhn & Meyer, 2009). This is likely due to the elusive nature of the Eurasian otter (*Lutra lutra*) that makes difficult the observation of wild individuals. This is true especially where population densities are low, as for the small population that recently colonized the River Sangro (Abruzzo region, Italy; De Castro & Loy, 2007), at the northern boundary of the species Italian range. As part of a Non Invasive Genetic Sampling of otters in this area we tested the efficiency of regular and sensor modified camera traps.

Aims

The principal aim of our study is to test the efficiency of camera trapping with Eurasian river otter in the wild, and specifically test weather body temperature could be responsible for the low detectability of the species.

Methods

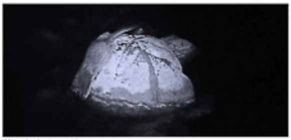
In the first step of our study we used two Scoutguard SG550, triggered camera equipped with a PIR sensor. Due to the uncertainty of slides really used by otters and accounted for their habits of swimming and marking, we decided to set camera traps near marking sites, *i.e.* rocks placed in the middle of river stream.

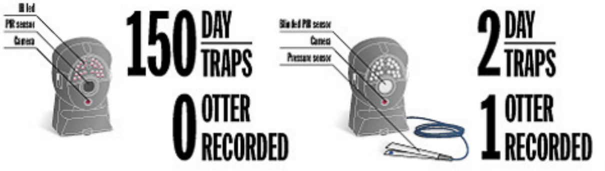
Cameras were set to record one minute videos or three consecutive photos. Camera traps were opportunistically checked daily or weekly. Their correct functioning and placement was confirmed by videos of other species at the same sites. The sensitivity was initially set on *"normal"* but after three occasions in which we found fresh spraints but no video recorded, we used *"high"* sensitivity.

Cameras were set both with and without odor baits (rotten fish scent).

After 150 day/traps we had no image of otters and consequently we hypothesized a detection problem due to the low temperature differential between the animal and the surrounding air, especially for animals emerging from the water to mark rocks in the middle of the river course. We suspected that for animals rapidly left ingwater, marking rocks and going back to the river, time could not be sufficient to warm their body and activate cameras.

We then modified one of the two Scoutguard SG550 by adding a pressure trigger instead of PIR. In this modified trap the PIR sensor was covered to guarantee the solely pressure activation.





Results

After 150 day/traps camera traps equipped with standard PIR sensor were not able to record any video of otter, despite the verified transit of at least one individual.

When we coupled the unmodified camera with the other provided with pressure triggers camera trap recorded a video of an otter after only two day/traps.

Discussion

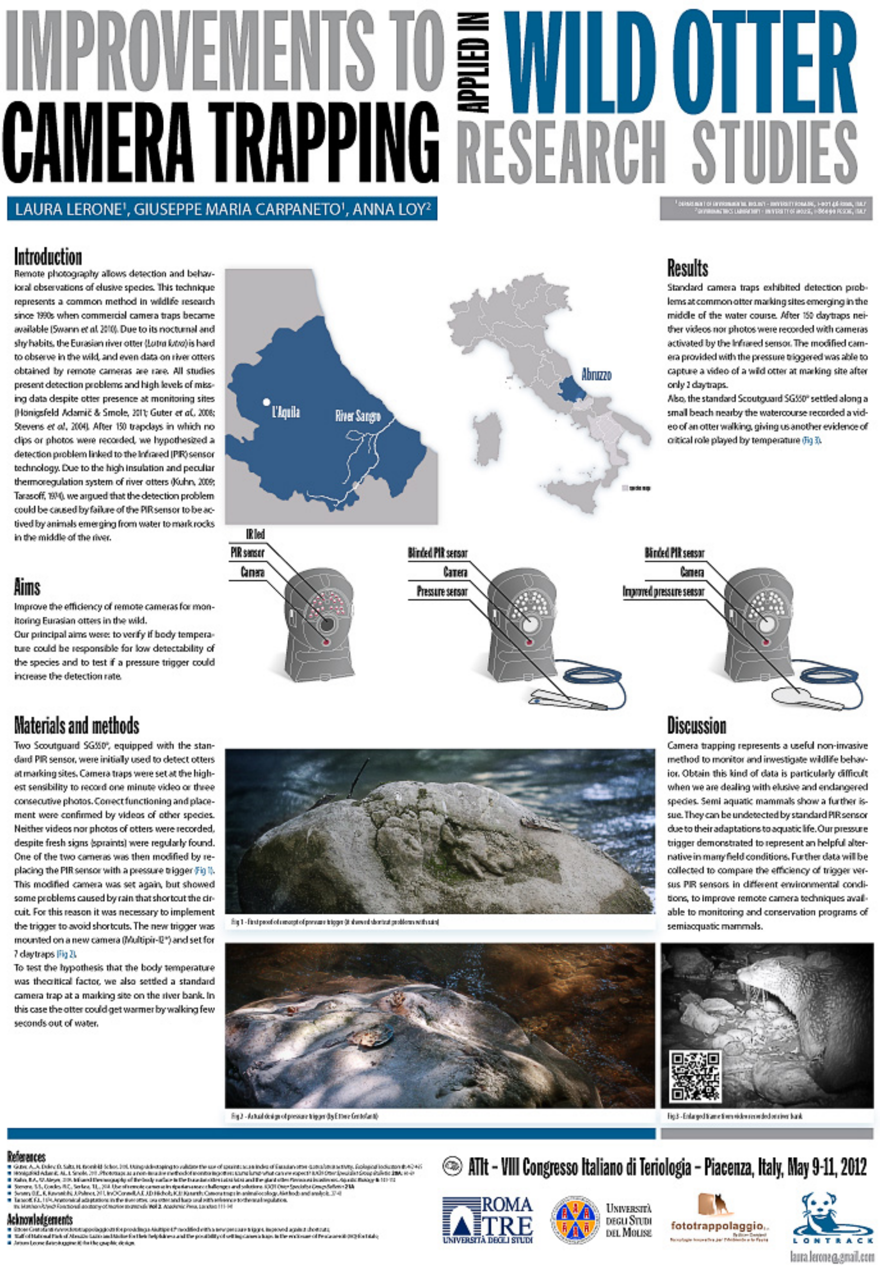
Despite further data are needed to confirm our outputs, our observations strongly support the hypothesis that common camera traps might often fail to catch otters as a consequence of their low body temperature when emerging from water. This is in accordance with recent studies conducted on otters using thermocams by Kuhn & *Meyer* (2009). Authors clearly showed that when otters leave water their temperature equals the water temperature. Due to the high adapted isolation and thermoregulation system of river otters (Tarasoff, 1974; Kuhn, 2009) there could be difficulties for standard PIR sensors of photo and video traps to activate cameras, especially when framing marking sites closed to freshwater courses. This represents a big deal for using camera trapping in otter's monitoring programs. Improving alternative techniques will give great chances in future research studies and conservation projects.

Limits and future improvements

Future improvement of the system will be devoted to solve the short-circuit of the pressure sensors caused by the rain. But we are confident there will be improvements in our method.

Acknowledgements

References



IMPROVEMENTS TO CAMERA TRAPPING APPLIED IN WILD OTTER RESEARCH STUDIES

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Introduction

Remote photography allows detection and behavioral observations of elusive species. This technique represents a common method in wildlife research since 1990s when commercial camera traps became available (Swann *et al.* 2010). Due to its nocturnal and shy habits, the Eurasian river otter (*Lutra lutra*) is hard to observe in the wild, and even data on river otters obtained by remote cameras are rare. All studies present detection problems and high levels of missing data despite otter presence at monitoring sites (Hönigsfeld Adamič & Smole, 2011; Guter *et al.*, 2008; Stevens *et al.*, 2004). After 150 trapdays in which no clips or photos were recorded, we hypothesized a detection problem linked to the Infrared (PIR) sensor technology. Due to the high insulation and peculiar thermoregulation system of river otters (Kuhn, 2009; Tarasoff, 1974), we argued that the detection problem could be caused by failure of the PIR sensor to be actived by animals emerging from water to mark rocks in the middle of the river.

Aims

Improve the efficiency of remote cameras for monitoring Eurasian otters in the wild.
Our principal aims were: to verify if body temperature could be responsible for low detectability of the species and to test if a pressure trigger could increase the detection rate.

Materials and methods

Two Scoutguard SG550®, equipped with the standard PIR sensor, were initially used to detect otters at marking sites. Camera traps were set at the highest sensibility to record one minute video or three consecutive photos. Correct functioning and placement were confirmed by videos of other species. Neither videos nor photos of otters were recorded, despite fresh signs (spraints) were regularly found. One of the two cameras was then modified by replacing the PIR sensor with a pressure trigger (Fig 1). This modified camera was set again, but showed some problems caused by rain that shortcut the circuit. For this reason it was necessary to implement the trigger to avoid shortcuts. The new trigger was mounted on a new camera (Multipir-12®) and set for 7 daytraps (Fig 2).
To test the hypothesis that the body temperature was thecritical factor, we also settled a standard camera trap at a marking site on the river bank. In this case the otter could get warmer by walking few seconds out of water.

Fig 1 - First proof of concept of pressure trigger (it showed shortcut problems with rain)

Fig 2 - Actual design of pressure trigger (by Ettore Centofanti)

Results

Standard camera traps exhibited detection problems at common otter marking sites emerging in the middle of the water course. After 150 daytraps neither videos nor photos were recorded with cameras activated by the Infrared sensor. The modified camera provided with the pressure triggered was able to capture a video of a wild otter at marking site after only 2 daytraps.
Also, the standard Scoutguard SG550® settled along a small beach nearby the watercourse recorded a video of an otter walking, giving us another evidence of critical role played by temperature (Fig 3).

Discussion

Camera trapping represents a useful non-invasive method to monitor and investigate wildlife behavior. Obtain this kind of data is particularly difficult when we are dealing with elusive and endangered species. Semi aquatic mammals show a further issue. They can be undetected by standard PIR sensor due to their adaptations to aquatic life. Our pressure trigger demonstrated to represent an helpful alternative in many field conditions. Further data will be collected to compare the efficiency of trigger versus PIR sensors in different environmental conditions, to improve remote camera techniques available to monitoring and conservation programs of semiacquatic mammals.

Fig 3 - Enlarged frame from video recorded on river bank

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Acknowledgements

ATIt – VIII Congresso Italiano di Teriologia – Piacenza, Italy, May 9-11, 2012

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NON-INVASIVE GENETIC SAMPLING OF THE EURASIAN OTTER IN ITS ITALIAN NORTHERN RANGE

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Introduction

The project concerns the status of Eurasian otter (*L. lutra lutra*) population in its northern limit of distribution in Italy. The Sangro basin in Abruzzo and Molise regions represents a critical zone for the viability and the future expansion of species in its former range, but scanty informations are available about this area recently recolonized by otters. The Eurasian otter is an elusive nocturnal mustelid with high conservation interest in Italy, where the species did not still recover after a bottleneck occurred in the 1970s. The main tool to assess population status in endangered and elusive species is the non-invasive genetics sampling (NGS), collecting biological samples like scat or hair directly in field. DNA in otter spraints is apparently exposed to very rapid degradation processes (Dallas *et al.*, 2003; Hajkova *et al.*, 2006) and success rate in DNA analysis is much lower than in other carnivores.

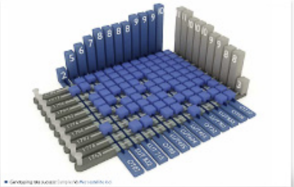
Aims

The main goal of this project is the study of the Sangro's basin otter population using microsatellites (Short Tandem Repeats, STRs) as molecular markers to analyse DNA. NGS allows to estimate minimum population, mean home range size and, depending on the number of samples, to infer the kinship between individuals. These informations are necessary to plan future monitoring and conservation programs for the species.

TAB 1	Number of alleles	Expected Heterozygosity	Observed Heterozygosity
LUT435	3	0,23	0,20
LUT604	3	0,53	0,60
LUT701	2	0,40	0,30
LUT715	3	0,40	0,10
LUT832	3	0,62	0,30
LUT833	6	0,72	0,60
LUT453	3	0,57	0,50
OT05	2	0,49	0,20
OT17	4	0,61	0,40
OT04	2	0,44	0,60
OT22	2	0,43	0,30
OT07	2	0,50	0,20
Mean	2,9	0,50	0,36

TAB 2	PID	PID(sibs)
LUT833	1,26E-01	4,29E-01
LUT832	2,73E-02	2,09E-01
OT17	5,68E-03	1,04E-01
LUT453	1,47E-03	5,51E-02
LUT604	4,18E-04	3,06E-02
OT07	1,56E-04	1,82E-02
OT05	5,94E-05	1,09E-02
OT04	2,42E-05	6,86E-03
OT22	1,01E-05	4,39E-03
LUT715	4,00E-06	2,84E-03
LUT701	1,78E-06	1,87E-03
LUT435	1,07E-06	1,48E-03

- Probability of Identity. Multi-locus PID(biased) and PID(sib) [illegible] of single-locus values [illegible]



- Genotyping success [illegible]

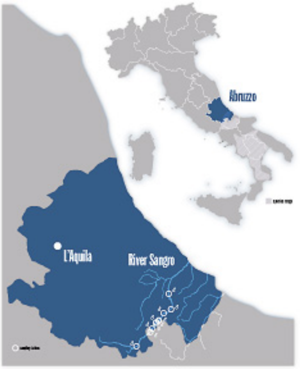
Methods

Sampling was carried out from May to October 2010 in ten marking sites along river Sangro. Sampling stations were cleared and then checked daily to find fresh spraints or jellies. Samples were sent to ISPRA Genetic Laboratory (Ozzano dell'Emilia, Bologna) within 3-20 days from sampling and then DNA was extracted. All samples were stored in ethanol 98% until DNA extraction, carried out with a Quick-gDNA™ MiniPrep Kit (Zymo Research). DNA was initially amplified with simplex PCR and 12 microsatellites loci were assayed: Lut435, Lut604, Lut701, Lut715, Lut832, Lut833, Lut453, OT05, OT17, OT04, OT22, OT07 (Chuan-Chin Huang *et al.*, 2005, Dallas and Piertney, 1998). The amplified loci were analysed on an ABI 3130 automatic sequencer (Applied Biosystems). Due to low quality and amount of DNA in otter spraints, a new analysis approach with nested PCR was carried out for every locus, obtaining a slight improvement of the amplification success rate. Samples were also subjected to WGA (Whole Genome Amplification) but the procedure did not lead to significant changes in amplification results. Zink-finger protein genes ZFX/ZFY were used for molecular sexing of individuals (Mucci *et al.*, 2007).

Softwares used to process genetic data are: *GeneMapper v.3.0* (Applied Biosystems ABI), a software used to correct results of automated analysis; *GenAlex v.6.0* (Peakall & Smouse, 2006) a software useful to estimate allele frequency, observed (HO) and expected unbiased (HE) heterozygosities, mean number of alleles per locus (NA), to perform genetic distance and assignment tests (through Principal Coordinate Analysis PCA) and several other parameters; *Gimlet v.1.3.3* (Valière, 2002) allows to identify genotypes described by multilocus microsatellite loci, to estimate the frequency of ADO (Allelic Dropout) and FA (False Alleles) by comparing results of individual replicate, to analyse data and produce estimates of several genetic parameters.

Results

WGA seems to be ineffective in increasing otter's DNA, due to the presence of a large amount of prey's DNA that was preferentially amplified by this technique. On the contrary, nested PCR was an effective tool to obtain higher quantities of target DNA. The period spent from sampling to extraction is one of the most important factors affecting success of PCR amplification (Hájková *et al.*, 2006; Lampa *et al.*, 2008). Ten of 22 samples passed the quality screening with three loci and were analysed at all loci. Because of the low quality of the DNA analysed, it was not possible to obtain complete and reliable genotypes of samples and therefore it was not possible individuals identification. All the analysed loci were polymorphic, the number of alleles per locus ranged between 2 and 6, with a mean number of alleles per locus of 2,9 (TAB 1). From molecular sexing, 8 samples were identified as belonging to males and 2 samples to females. Nevertheless it was not possible to distinguish between individuals, so we can only be sure of the presence of a minimum of two individuals in the study area, one male and one female. Heterozygosity (TAB 1) didn't show high values (Ho= 0,36), and low variability in Italy could be explained either by post glacial founder event or a more recent population decline. TAB 2 reports Probability of Identity (PI) results. Errors in individual identification can be minimized by increasing the number of informative microsatellites and obtain lower PID (Probability of Identity) values. In this study we used two PID formulations (Waits *et al.* 2001): PID_{unb} = unbiased for small sample size, and PID_{sibs} = the expected PID between sibs, which define respectively the lower and upper bounds assuming that the sampled populations included only unrelated individuals or sibs. PID values were estimated for 12 loci using GIMLET in the 10 non-invasive genotypes identified.



TAB 3	Positive PCR among Locus	Dropout	False Alleles
LUT435	0.70	0.83	0.00
LUT604	0.77	0.00	0.08
LUT701	0.57	0.67	0.07
LUT715	0.33	0.00	0.00
LUT832	0.40	0.00	0.00
LUT833	0.63	0.00	0.07
LUT453	0.33	0.00	0.10
OT05	0.40	0.00	0.13
OT17	0.70	0.17	0.00
OT04	0.73	1.00	0.11
OT22	0.70	0.50	0.07
OT07	0.10	0.00	0.00

- PCR success rate/N° per locus

TAB 4	Positive PCR among samples	Dropout	False Alleles
1763	0,11	0,00	0,00
1766	0,50	0,00	0,03
1774	0,39	0,11	0,03
1776	0,44	0,00	0,08
1777	0,78	0,00	0,12
1782	0,42	0,00	0,08
1783	0,64	0,10	0,03
1784	0,75	0,00	0,07
1785	0,81	0,44	0,13
1786	0,47	0,00	0,00

- PCR success rate/N° per sample

Discussion

This pilot study permitted us to refine sampling and analysis protocols for molecular analysis of otter spraints. The project will be extended to a broader study area along river Sangro and its tributaries; the collection effort will be maximized to get a higher number of samples. DNA extraction will be performed immediately after sampling to minimize degradation and to increase amplification success. QIAamp DNA Stool Kit (Qiagen) will be used for DNA extraction to improve amplification rate (Lampa *et al.*, 2008).

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Acknowledgements

- ISPRA Genetic Laboratory staff (Ozzano dell'Emilia, Bologna) for [illegible]
- PhD Lorenzo Quaglietta for his constant support, [illegible] for the cooperation.
- [illegible] for the graphic design.
- Prof. [illegible] for the logistic support.

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GENETIC STRUCTURE AND INDIVIDUAL IDENTIFICATION OF EURASIAN OTTER POPULATION (*LUTRA LUTRA*) ALONG RIVER SANGRO BASIN

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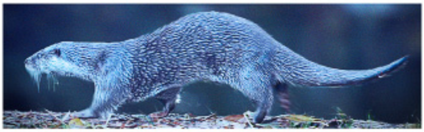
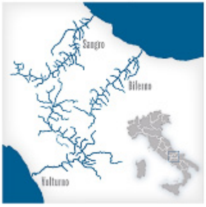


Introduction

To monitor and manage endangered species we firstly need basic information on population status and distribution. When dealing with elusive and protected species, one of the most useful and feasible approach is the non-invasive genetic sampling (NGS). Due to its high elusiveness, nocturnal habit and marking behavior, the otter represents a good target species to apply NGS. Nevertheless DNA in otter scats (spraints) appears exposed to a very quick degradation process. Consequently the DNA amplification success rate is much lower than in other carnivores (Dallas *et al.*, 2003; Hajkova *et al.*, 2009). We tried to implement the available protocols for DNA analysis from otter spraints in a study on the otter population living in the river Sangro (Abruzzo, Italy) . For the first time in Europe we used 7 microsatellite loci (OT), designed for *L. lutra* in Asia, for a total 13 microsatellite loci as genetic markers.



Aims

Our main goal is the genetic characterization of the Sangro's basin subpopulation in order to infer the minimum number of individuals, genetic variability, and genetic similarity with otters living in the neighbouring Volturno and Biferno basins.

Materials and methods

Otter scats were collected from April to September 2011 along river Sangro basin, Volturno and Biferno. Marking sites, detected during a pilot study, were checked for fresh spraints and anal jellies. Daily checks guaranteed the collection of samples within 24 hours from deposition. Samples were collected and stored in ethanol 96% at -20°C. The analysis encompassed a panel of 13 microsatellite loci (Lut453, Lut833, Lut832, Lut604, Lut701, Lut902, Ot04, Ot05, Ot07, Ot17, Ot22, Ot19, Ot14) and ZFX/ZFY sequences for sex identification (Mucci & Randi, 2007; Huang *et al.*; 2005, Dallas *et al.*, 2003). To minimize errors and costs, all samples were initially screened for three microsatellite loci (OT07, OT14, OT17) with four independent repeats per locus. Only samples with 50% or more positive PCR were analyzed at all microsatellite loci. To allow the detection of dropouts and false alleles we used a multiple-tube approach (Taberlet *et al.* 1996), where the same DNA sample was amplified independently several time per locus. Negative controls were included in each extraction and PCR to monitor contamination. GeneAlex® and GIMLET® (Valière, 2002) were used to descriptive analysis of genetic data.

Results

A total of 266 fresh samples were collected, including spraints, jellies and jellies mixed to food remains. 35 samples were collected twice in order to compare different extraction kits or storage buffer solutions (results are not included). Of the 231 total unique samples 184 belonged to the Sangro and 47 to the Biferno and Volturno basins. The total genotyping success rate was 34,2%. Pure jellies showed 76,9% of success while spraints mixed to jellies 28,0% and spraints 25,6%. The mean frequency of allelic dropout (ADO) among the Sangro's samples was 28,6% (SD=0,06) among loci, and 14,7% (SD=0,05) among samples. Expected probability of identity values (PI and PI_{sibs}) are presented in Fig 1.

Tab 1 reports synthetic data of ADO, false allele (FA) and Positive PCR. We assessed the presence of at least six individuals along the river Sangro and its tributaries. The number of genetic recaptures ranges between 1 and 19. We also identified three distinct individuals from samples belonging to Biferno and Volturno basins with recaptures range between 1 and 4. Analysis on molecular sexing is in progress. Alleles, frequencies and private alleles for the two subpopulations are presented in Tab 2.

Locus	N_a	H_o	H_e	% ADO	% FA	% PCR+
LUT701	3	0,43	0,53	25,5	0,6	70,0
OT19	4	0,46	0,53	27,9	0,9	70,0
OT17	3	0,58	0,53	27,8	4,4	81,0
LUT833	3	0,92	0,53	21,8	1,4	87,0
OT22	3	0,56	0,48	19,5	0,4	91,0
OT04	2	0,77	0,50	29,9	0,0	76,0
LUT832	2	0,44	0,43	27,5	0,0	81,0
OT05	2	0,45	0,42	22,6	0,0	80,0
OT14	2	0,42	0,41	14,1	0,3	90,0
LUT604	2	0,55	0,40	20,9	0,0	94,0
LUT902	2	0,50	0,37	24,8	0,4	87,0
OT07	2	0,47	0,37	21,8	0,0	80,0
LUT453	1	0,00	0,00	14,8	0,8	86,0

Tab 1: Number of alleles (N_a), observed (H_o) and expected (H_e), percentage of allelic dropout (% ADO), percentage of false alleles (% FA) and percentage of positive PCRs (Samples from Sangro basin)

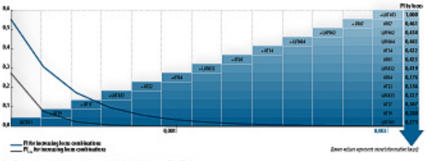


Fig 1: Theoretical expected probability of identity values based on allele frequencies (PI and PI_{sibs})

Locus	Allele	Sangro	Volturno Biferno
LUT832	184	0,000	0,316
	188	0,689	0,526
	192	0,000	0,158
	196	0,311	0,000
LUT902	147	0,750	0,737
	151	0,250	0,263
OT04	176	0,538	0,500
	204	0,462	0,500
LUT453	125	1,000	0,833
	133	0,000	0,167
LUT604	129	0,727	0,632
	131	0,273	0,368
LUT701	202	0,169	0,105
	206	0,631	0,763
	218	0,200	0,132
OT05	175	0,697	0,395
	179	0,303	0,605
LUT833	147	0,538	0,053
	155	0,424	0,447
	163	0,038	0,500
OT07	200	0,750	0,632
	204	0,250	0,368
OT17	145	0,876	0,059
	153	0,606	0,618
	157	0,318	0,324
OT14	116	0,000	0,158
	120	0,712	0,526
	124	0,288	0,316
OT19	211	0,085	0,029
	215	0,246	0,471
	219	0,000	0,029
	223	0,034	0,176
	227	0,636	0,294
OT22	148	0,636	0,553
	152	0,341	0,316
	164	0,023	0,132

Tab 2: Microsatellite loci, alleles and allele frequencies. In Blue private alleles and their frequencies

Discussion

The new protocol allowed to identify at least nine individuals of otters living in the northern portion of the otter range in Italy. More accurate data were gathered on the river Sangro, were at least six otters are actually present. These results suggest a stable presence of the species, that was first recorded in the river in 2007, after being extirpated in the '90s (De Castro & Loy, 2007). This is also the most northern known area of occurrence of the otter in Italy (Panzacchi *et al.*, 2011), and therefore this river basin is a strategic and crucial area for the future expansion of the species in Italy. Analyses in progress will likely allow to identify the source population from the neighbouring river basins Volturno and Biferno, as well to define the population structure. We emphasize two critical steps: a careful sampling in the field and a close analysis of potential genotyping errors in laboratory. Consistent data on wild otters are vital for conservation purpose and noninvasive genetics represents a powerful tool for obtaining reliable data.

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Acknowledgements

- [illegible] for the logistic support.
- [illegible] for the graphic design.

ATIt - VIII Congresso Italiano di Teriologia - Piacenza, Italy, May 9-11, 2012

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Acknowledgements

Support, Funds and logistics

I intensely thank

Prof. **ANNA LOY** (University of Molise) for the crucial support all over the Ph.D. and to provide many field work facilities and congresses fees

Prof. **ETTORE RANDI** (ISPRA) for providing laboratory facilities and logistics, for supporting most of the costs of genetic analyses and for his experienced and essential advices

ETTORE CENTOFANTI to fund my project with four camera traps provided with modified pressure triggers (www.fototrappolaggio.com)

Dr. **CINZIA SULLI** (PNALM) for providing research permissions in the National Park of Abruzzo, Lazio and Molise and for permitting me to set camera traps in the PNALM’s otter fence

Prof. **PAOLO CIUCCI** (University of Rome “Sapienza”) for providing me the deep freezer in which I stored all otter samples in Abruzzo

Ph.D. **CHIARA MENGONI** (ISPRA) for the quick DNA extraction of our “horrible” samples, the constant support and the extraordinary laboratory training

Further acknowledgements

ARTURO LEONE for the shared life, patience, countless advices and support in any phase of the project.. and for all the Ph.D. graphics

CARLO LERONE and **GIULIANA SIMONETTI** (my parents) for supporting me in any choice (almost any) ever

CHIARA and the others ISPRA guys (**ALESSANDRA**, **ELENA**, **FEDERICA**, **FRANCESCA**, **MARCO**, **NADIA** and **ROMOLO**) for sharing laughs and for answering all my continuous questions

Prof. **MARIA LINA BERNARDINI** and Ph.D. **LUIGI LEMBO FAZIO** (University of Rome "Sapienza") for their partnership in bacterial *spraint* analyses

Dr. **DAVIDE RIGHETTI** and Dr. **LUCA LAPINI** to provide me with informations with the current status of otters in North-East Italy

Prof. **JUAN PABLO GALLO REYNOSO** and Ph.D. **LORENZO QUAGLIETTA** for sharing with me their passion and knowledge about the wonderful world of otters. Lorenzo also for encouraging me to apply for a Ph.D. position

JANITZIO, **ELSA**, **"CHUITO"** and **MARICARMEN** for their friendship (better brotherhood!) during the first field experience with the beast (*Lontra longicaudis*) and from then on! I also thank all my Mexican and almost Mexicans friends (**ALICE**, **ANDROMEDA**, **CHIARA**, **DAVIDE**, **ELENA(1,2)**, **FRANCESCA**, **FRANCESCO (2,3)**, **L'ELISA**, **LEO**, **MARTA**, **MERIENGEL**, **MERIO**, **MIKI**, **PAOLINO**, **PIETRO**, **SARA**, **SILVIA**, **SILVIO**, **VIVI**) for waiting patiently my long disappearances

ALICE BRAMBILLA for the crucial brainstorming for the Lontrack naming

FRANCESCO IMPERI and **GIULIA CAPOBIANCO DONDONA** for choosing Lontrack project for their theses and for giving me a true help

MERY LERONE for inciting me when I said "I will never work in genetics!"

All my helpers who made the field work less hard (sometimes more): **ALESSANDRO**, **ALESSIA**, **FLAMINIA**, **FRANCESCO**, **GABRIELE**, **GIULIA**, **LAURA**, **LAVINIA**, **PIETRO**, **SIMONA**, **VALERIA**, **VITTORIA**

DR. GIUSI DE CASTRO for Biferno and Volturno otter samples

FRANCHINO for revealing me some secret otter's places with his over 70 years of Sangro experience

FRANCESCO the best auto mechanic off Mother Russia

"Casale" guys (**CORRADO**, **IVAN**, **LEO**, **MARTINO**, **PUPI**, **RITA**, **SILVIA**, **TEO**) for hosting me and sharing their complex lives

FAUSTO, **MARCO**, **ORLANDO**, **DANIELA**, **ELISABETTA**, **MARIA**, and the other "boitans" for the shared funny and philosophical moments during the long seasons in Abruzzo

ALESSANDRO and **DAVIDE** for the best barbecues ever tasted

LUISA for the continuous encouragement despite the adversities

ADRIANO for sharing an open mind point of view and for all caffeine doses

My historical friends **GIULIA** and **LUIGI**, so far and yet so close

My **LADA** for taking me anywhere I wanted and always back home

WHO gave me the rage.