BEST PRACTICE ACTIONS FOR WOLF CONSERVATION IN MEDITERRANEAN-TYPE AREAS

Action A.2

Ex-ante detailed survey of wolf presence in the Portuguese project areas

Final Report

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1. State of the Art

The first national wolf survey conducted by Grupo Lobo and the Nature Conservation Institute (now ICNF) in 1995-96 (ICN 1997) identified two wolf packs in the project's study area (East of the Côa River), belonging to the wolf nucleus located South of the Douro River, and defined its population status as unstable and threatened. A second national wolf survey made in 2002-03 (Pimenta *et al*. 2005) confirmed wolf presence in 2 UTM (10x10 km squares) and 2 home ranges of "probable" wolf packs.

In 2011-2012, the most recent wolf study conducted in the region by Grupo Lobo and Zoo Logical, described an increase in the species range (8 UTM) and identified one potential wolf pack (West of the Côa River) (Cadete *et al.* 2012). These data might indicate a natural recolonization process, but results could have been biased by the successful application of new methods such as non-invasive genetic sampling (NGS) and camera trapping, as these techniques can improve the accuracy of wolf detection. During this study, no reproduction was detected although there was a camera-trap record of 2 adult wolves in a joint attack to livestock. A dead female was found in the northern range of the project's study area with undetermined cause of death.

Non-invasive genetics is based on genetic identification of species, individual genotype and sex of unknown biological samples (scats, urine, saliva and hairs), which are collected without any interaction with the animals, and has been successfully applied in monitoring programmes of elusive species like the wolf (Fabbri *et al.* 2007; Lucchini *et al.* 2002; Marucco *et al.* 2009; Galaverni *et al.* 2012). Microsatellites (STRs) have been the marker of choice in the last two decades, used to identify species and to detect individual genotypes from non-invasive and forensic samples (Taberlet & Luikart 1999; Broquet *et al.* 2007). However, DNA degradation of these kind of samples leads often to low amplification rates and genotyping errors (Bonin *et al.* 2004). These errors can be minimized by a multiple-tube replication approach (Taberlet *et al.* 1997) and by statistical validation of the results of PCR replicates (Miller *et al.* 2002; Valière 2002) making the non-invasive genetics a method that can provide data that could not be obtained by any other monitoring method (Waits & Paetkau 2005).

At INIAV (Instituto Nacional de Investigação Agrária e Veterinária), non-invasive genetics monitoring is being carried out for more than 8 years, using microsatellites as molecular markers. The same markers used in the present study led to the identification of different wolf genotypes that were sampled in a particular Portuguese geographical area North

of the Douro River from 2008–2012 (Borges *et al.* 2012). In one of these areas, the five different wolf genotypes were identified and found to agree in number with monitoring data from camera trapping methods (Ferrão da Costa *et al.* 2012). Furthermore, forensic analysis from livestock attacks was performed, showing a success rate of around 42% (Borges *et al.* 2012a). NGS using microsatellite marker allowed also to estimate a minimum population size of 8 individuals West of the Côa River, during the above mentioned wolf survey.

2. Goal

The objective was to conduct an accurate wolf survey using non-invasive methods, including direct and indirect field techniques and genetic analysis. Specifically we sought to: produce a reliable and updated wolf distribution map; identify potential wolf packs; estimate the minimum population size; and understand demographic patterns that might influence population trend.

3. Study Area

The study area (5026 km^2) (Fig. 1) is located in the centre of Portugal, south of the Douro River, in the bordering region with Spain. It consists of a plateau (300-900 m high) with typically Mediterranean habitat features including flora species such as the Holm oak (*Quercus ilex*), the Gum rockrose (*Cistus ladanifer*) and emblematic endangered avifauna like the Egyptian vulture (*Neophron percnopterus*) and the Black stork (*Ciconia nigra*). Its highly humanized landscape mainly consists of agricultural patches mixed with forested areas scattered by small villages. It covers 7 municipalities and 4 protected areas (Fig. 2): Estrela Mountain Natural Park, Malcata Mountain Natural Reserve, International Douro Natural Park and International Tejo Natural Park.

It also includes one Natura 2000 site (Malcata) and one private natural reserve (Faia Brava). The river network includes Rivers Douro, Tejo, Águeda, Côa and the Cabras's Creek. Estrela, Marofa and Malcata are the main mountain like landscape throughout the region. Although village numbers are high (INE 2011), it is in general a low human density territory (INE 2011), where farming and husbandry are the main economic activities. Livestock is commonly grazed in large poorly fenced areas that include pastures, bush and forested clusters. Semi-free range cattle, raised for meat production, and sheep and/or goat flocks are seldom watched by shepherds or guarded by dogs.

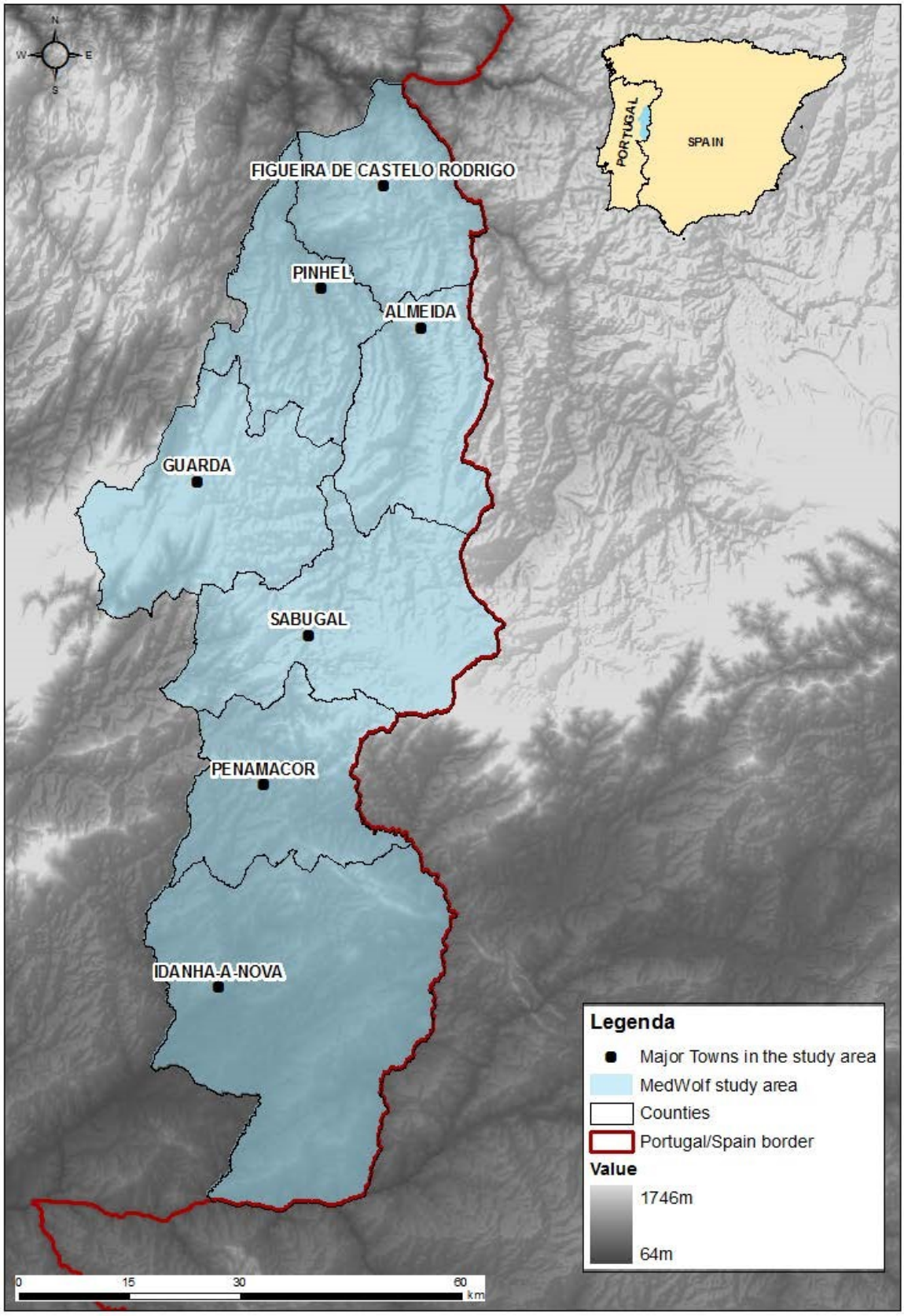

Figure 1 – Municipalities in the study area.

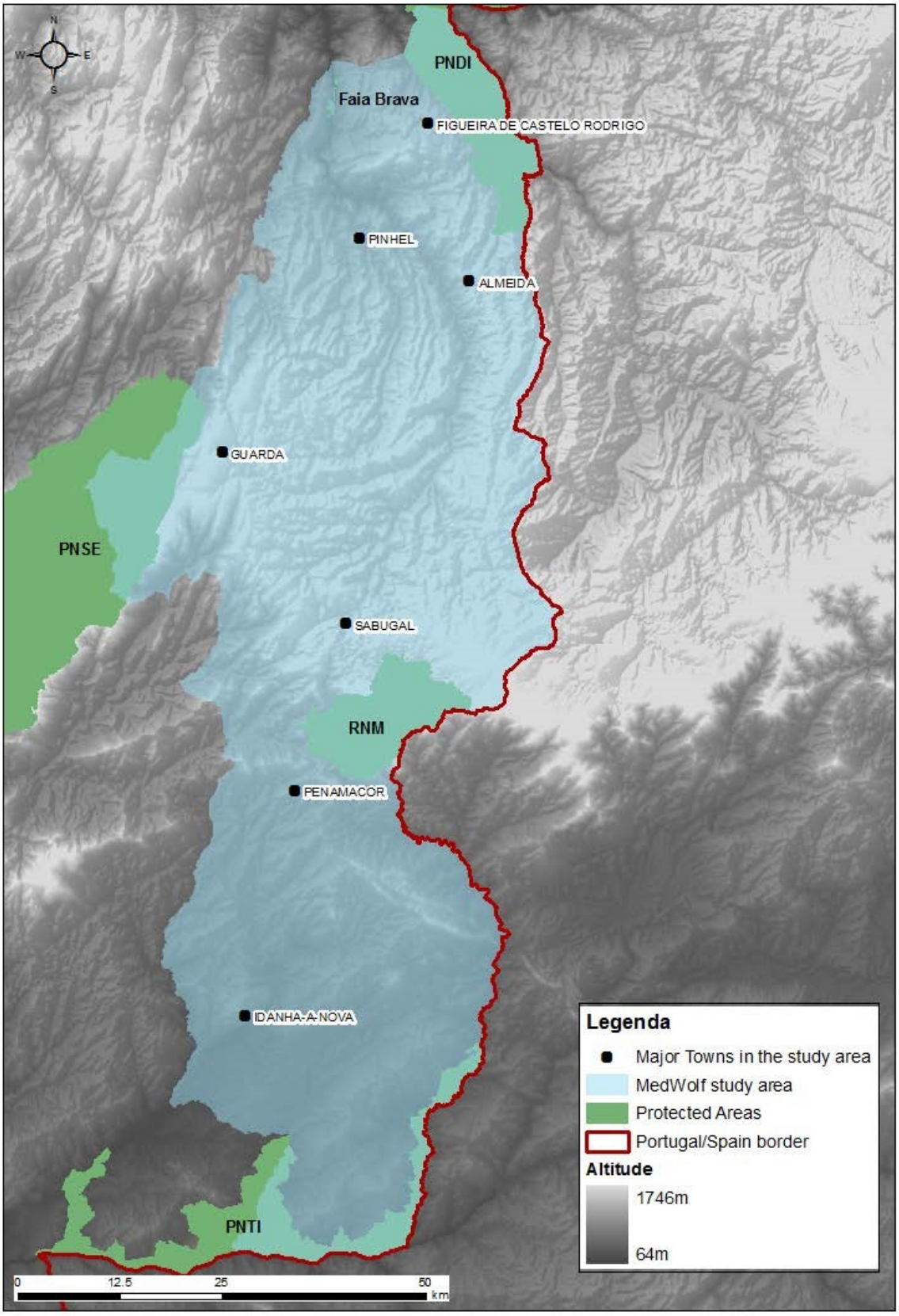

Figure 2 - Protected areas located in the study area (Estrela Mountain Natural Park - PNSE, Malcata Mountain Natural Reserve - RNSM; International Tejo Natural Park – PNTI), and a private natural interest area (Faia Brava Reserve).

Three wolf wild prey species occur throughout the study area: wild boar (*Sus scrofa*), whose density is perceived as high, even though regional studies are missing , red deer (*Cervus elaphus*) (only in the southern range and mainly in game hunting areas) and roe deer (*Capreolus capreolus*), which population, despite being expanding (Salazar 2009), is still not well known. Sport hunting (especially wild boar and rabbit) is popular throughout the study area. Free-ranging dogs are somewhat common in the study area, resulting from abandoned livestock guarding or hunting dogs. They can even form organized "dog packs" and inflict damages in domestic animals, posing serious problems for wolf monitoring and management, since they make it more difficult for the correct identification of the predator responsible for damage on livestock as well as for scat sampling by wolf biologists.

4. Material and Methods

4.1. Fieldwork

Fieldwork was conducted from the 1st of December 2012 to the 30th September 2013, except for camera trapping, that was continued until the 31st October 2013.

4.1.1. Livestock damage forensic genetics

Reported canid (domestic or wild) attacks on livestock were forensically investigated throughout the study period, using molecular identification of the DNA present in hair or salivary samples collected from carcasses or in kill sites (see 4.4. for detailed DNA analysis). In each occurrence, a minimum of three salivary swab samples were collected from visible bite wounds and consumed body parts (Fig. 3).

A thorough search of the surroundings was made to look for scats or hair. Using the carcass as a centroid, an exhaustive examination of a 100m radius around it was done, looking for wolf evidence, namely scats. In wired-fence explorations, a search for wolf hair was conducted, especially in areas that could be used as fauna trail.

Figure 3 – Swab sampling of a bite wound in a sheep carcass.

4.1.2. Interviews to local people

Interviews (n=70) were opportunistically conducted throughout the study area to gather information about wolf sightings, breeding, mortality and livestock damage. These interviews were complemented with data specifically gathered during the systematic interviews conducted in Action A11 (n=222), developed from February to June 2013, mainly to hunters, livestock owners/shepherds and forest rangers, where the same subjects were inquired (beside other relevant issues).

The informants were judged reliable if they were considered to have some knowledge and experience with wolves (e.g. hunters, forest rangers or livestock breeders with frequent wolf damage, recognized by the official authorities) and if the observation conditions enabled a precise observation of the animal (e.g. light, duration). Furthermore, observations were considered in the analysis if they were confirmed by different observations of distinct observers. Nonetheless, informations were never considered as a confirmation of wolf presence, but only as an indication on probable presence to be verified by field data.

4.1.3. Wolf sign survey

Each of the 65 UTM grid squares (10x10 km) superimposed onto the study area were surveyed by travelling 2 pre-defined transects, with a minimum length of 2 km each. Transects design took advantage of agricultural and forest roads and were selected on the

basis of quality regarding wolf potential habitat as well as low human presence (whenever possible). They were covered mainly by car (max. speed 10 km/h) with walking inspections on primary road intersections, 100m each way. A total of 310.8 km were surveyed (average 4.8 km/square) searching for wolf tracks and mainly wolf scats for subsequent genetic analysis. Potential wolf scats were collected in plastic bags, catalogued with several parameters (e.g. geo-reference, surrounding habitat, age, atmospheric conditions), and then frozen until being delivered to the laboratory. The mean waiting time between scat collection and genetic analysis was 3 weeks.

4.1.4. Camera trapping

Camera trapping survey did not intend to provide wolf abundance index data. Ten to fifteen Bushnell TrophyCam cameras were placed on different locations (11 UTM) (Fig. 4) selected on the basis of previous and current information about potential wolf presence, namely historical data, recent damage locations and information from interviews. For this reason, and due to the available data, the use of this methodology was carried out only in the northern part of the study area, since in the southern part no information prompted the use of the cameras.

Camera-traps were used throughout the study period (2,470 trapping-nights), one per trapping station, mainly aiming at dirt roads or animal trails, with a 0.66 second trigger and 3 photos per event, and using bait (meat or fish) in some locations. This methodology intended to assess:

a) Wolf presence;

b) Solitary individuals and wolf packs (minimum pack size);

c) Reproduction occurrence (pups, juveniles, lactating or post-lactating females);

d) Wolf + dog pack presence.

Due to the considerably frequent human presence in the study area, most baited cameras were under covered in bush or forested areas, to minimize the risk of being stolen. Because of this, baited camera-traps were primarily used to maximize data collection.

4.1.5. Direct methods

These methods presuppose some form of direct contact with the wolves, that in our case were auditory (howling sessions) or visual (watching sessions). They intend to: a) locate and identify wolf packs; b) assess minimum pack size and structure; c) assess breeding success; d) locate the den and rendezvous sites.

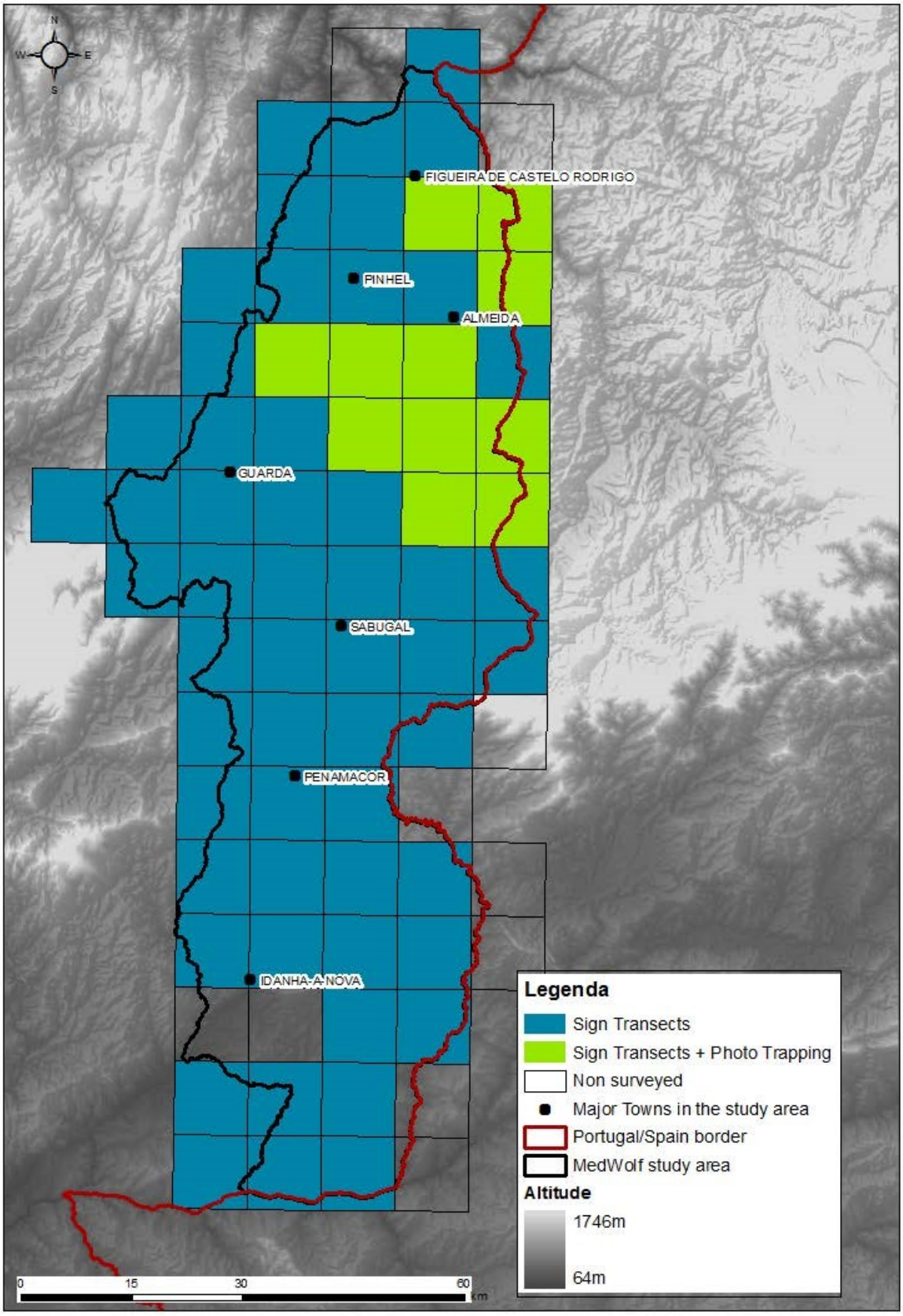

Figure 4 – Wolf sign survey and camera trapping in the study area, mapped onto the UTM grid.

Howling sessions consists in simulating howling to induce a reply by the wolves and with that confirm their location, minimum number and the presence of pups. They are conducted preferentially 1-2 hour after sunset, from high places near suitable habitat, selected on the basis of previous positive results from indirect methods, such as sign survey, camera trapping and interviews. Human-simulated howls are produced at specific sites and surveyors listen for a specified amount of time (e.g. 10 minutes) for a response, according to the methodology described by Harrington & Mech (1982).

Watching stations are carried out also from high places with a good field view area, near places where howling stations had some positive results or where information about recent sightings occurs. Due to the low success rate of this method, especially if not conducted in areas with previous howling stations success, it has been replaced by passive camera-trapping.

Howling sessions (n=24) and watching sessions (n=1) were conducted opportunistically from August to September 2013 in areas consistent with the above specifications.

4.1.6. Mortality

Mortality data was gathered from interviews to shepherds, hunters and farmers and also from the official records available from the Dead Wolves Monitoring System (SMLM), managed by the ICNF. In the scope of this national and centralized System every wolf found dead in the Portuguese territory is collected by wardens from the ICNF being necropsied to assess the cause of death and determine physiological parameters, as well as collect biological samples that will be available to the scientific community to pursue diverse types of research (e.g. genetic analysis).

4.1.7. Scat detection dog team

Under the supervision of Dr. Heath Smith, the dog trainer from Conservation Canines (University of Washington), one dog was selected from public dog shelters and trained to detect Iberian wolf scats in the wild in the scope of A7. This dog was trained using captive and genetically confirmed wild Iberian wolf scat samples, adapting well known narcotic detection dogs training principles (box work) to this task. The training of the Dog Team (dog and handler) occurred during one month, from May to June 2013, and included several sessions in a high wolf density region (Bragança District) to increase training success and efficiency. After this intensive training period, the dog team kept practising under controlled environment using captive and wild samples and continued to train regularly in Bragança

District. Although improvement training was still being carried out until the end of the wolf survey period, the dog team conducted 4 short survey sessions in the study area. The dog surveys consist of foot transects on dirt trails, with a length of 1-2 km, where the dog was lead to find as much wolf signs as he could.

4.2. Official damage reports

Data from the ICNF wolf damage database was analysed, covering the period from January 2012 to September 2013. The information analysed included the number of attacks, number and type of killed, injured and disappeared animals.

4.3. Criteria and data analysis

Data analysis was based on the following criteria:

a) Samples (scat, hair, swab) where considered to belong to wolf (or dog) after successfully genetic species assignment;

b) An attack was considered to have been done by wolves if a minimum of one biological sample collected on site was genetically wolf confirmed;

c) An attack was considered to have been done by domestic dogs if only genetically confirmed dog samples were collected on site;

d) For wolf pack assessment, all "wolf positive" official damage reports were considered, even in cases where forensic analysis results were contrary (dog positive); in fact, frequently dogs are the last animals to be in contact with the carcasses, which makes their saliva and fluids to prevail over the ones of the original predators; other parameters were also taken into consideration (e.g. the type of wounds, the presence of scats, the dragging of the carcass, etc.).

f) Data records used to confirm wolf presence were: 1) direct observation by a team member; 2) howling detection by a team member; 3) photograph record; 4) identification with NGS; 5) mortality record;

g) UTM squares with wolf confirmed presence have a minimum of 1 wolf record obtained with f);

h) A wolf pack was considered "possible" if throughout its defined home range a minimum of 10 attacks were recorded and validated by the official authorities (from January to October) (during a minimal of 5 months and ≥1 attack/month; according to the criteria proposed by Pimenta *et al.* 2005 and based on a long term data evaluation made on average number of damages in monitored wolf pack in Portugal), implying regular wolf presence year round, and

genetically wolf confirmed data were collected during the study period;

i) A wolf pack was considered "confirmed" if one of the following criteria were met: 1) throughout its defined home range a minimum of 30 attacks were recorded and validated by the official authorities (from January to October) (during a minimum of 8 months per year, and ≥3 attacks/month; according to the criteria proposed by Pimenta *et al.* 2005 and based on a long term data evaluation on average number of damages in monitored wolf pack in Portugal), where the higher number of attacks per month implies regular presence of more than one individual year round, and wolf presence was genetically confirmed during the study period; 2) reproduction was detected; 3) direct observation or wolf howling records of more than one individual were registered; 4) camera trapping records of more than one individual were registered;

j) If pack size couldn't be assessed by field work, average pack size estimates followed the criteria defined in the last Portuguese wolf census (Pimenta *et al.* 2005) for confirmed packs in the wolf population south of the Douro River: a minimum 2 individuals (breeding pair) during Winter/Spring (April-June – before the breeding season) and a maximum of 7 individuals during Summer/Autumn (breeding pair and 5 pups – after the breeding season). These estimates, based on data gathered during several years of wolf monitoring in the region and comparisons with other studies in the Iberian Peninsula, are lower than the ones for the northern Douro wolf nucleus, since this population is marginal and has lower density, undergoing high human-caused mortality, and thus the number of wolves per pack is considered to be lower than in more stable and higher density populations.

4.4. Genetic analysis

4.4.1. DNA extraction

DNA Extraction from blood, tissue and hair samples

DNA was isolated using either standard methods (i.e. Chelex protocol, Walsh *et al.* 1991) for blood samples, or commercial kits (Qiagen, Hamburg, Germany and AnalytikJena, Jena, Germany) according to manufacturer instructions.

DNA Extraction from scat samples

Sample preparation and handling

Each scat was handled individually, in a dedicated room in order to prevent external contamination. From each sample, a portion of the outer layer (containing the mucosal layer)

in several regions of the scat surface, were retrieved using a sterile scalp and moved to a sterile eppendorf tube that was immediately stored at -20 ºC until DNA extraction. Each DNA extraction was accomplished using a batch of 11 samples and one blank control (empty kit column) using the QIAamp DNA Stool Kit (QIAGEN) with some modifications to the protocol instructions, in order to optimize the extraction efficiency. The starting quantity of biological material was dependent of the faecal sample quality, being higher in low quality scats (evaluated after visual analysis, as the environmental samples were collected after being exposed to different and unknown atmospheric conditions for an undetermined amount of time). DNA precipitation time, with ethanol at -20 ºC was increased up to several hours (vs 20 min.) to increase precipitation efficiency, and DNA elution was performed in a final volume of 180 µL. The Innuprep stool kit (AnalytikJena) was also used according to manufacturer instructions. All DNA samples were maintained frozen at -20 ºC until use.

Microsatellite PCR Amplification and genotyping

In order to avoid cross-contamination among DNA samples during extraction and PCR all reactions were prepared in a laminar flow hood using aerosol-resistant pipette tips and pre- and post-PCR experiments were carried out in separate rooms. In all cases, the occurrence of contamination events was monitored by including negative and positive controls in each PCR experiment. We used 19 canid nuclear microsatellite markers to amplify scat DNA in short amplicons: AHT121 (Holmes *et al.* 1995), C22.279, CXX.109, CXX.173, e CXX.225 (Ostrander *et al.* 1993), FH2001, FH2054, FH2247, FH2010, FH2159 (Francisco *et al.* 1996), FH2611, FH4012, FH3210, REN247M23 (Guyon *et al*. 2003) e PEZ06, PEZ08 (Neff *et al*. 1999), FH2361 (Mellersh *et al*. 1997), VWF.X (Shibuya *et al*. 1994), C38 (van Asch *et al*. 2009). All forward primers were fluorescently labelled (6-FAM, Hex, NED from Applied Biosystems). We conducted PCRs in seven multiplex groups using the Qiagen multiplex kit according to the manufacturers recommended protocols (including Q-solution). Products were electrophoresed on an ABI 310 capillary sequencer (Applied Biosystems, Foster City, CA, USA) and alleles scored relative to an internal size standard, Genescan 350 Rox (Applied Biosystems), using GeneScan v3.7. Non-invasive genotypes were validated by replicated independent PCRs until each allele was observed at least twice. To summarise, the analysis proceeded as follows: (1) all DNA samples underwent PCR amplifications under standard conditions at all loci (if no product was detectable at one locus, the PCR was repeated up to 4-5 times), (2) PCR products and microsatellite profiles were checked for quality and rated.

4.4.2. Molecular sexing

The sex of each sample was identified by amplifying a fragment from SRY, a Y-linked gene according to Olivier *et al.* (1999).

4.4.3. Data analysis

Standard measures of genetic diversity and principal component analysis (PCA) were calculated in GenAlEx 6.5b (Peakall & Smouse 2006) using a reference set of 131 dogs (autochtonous breeds and stray dogs from Portugal) and 92 Iberian wolf genotypes obtained from tissue samples. To determine genetic structuring and individual assignments based on the autosomal microsatellite data set, we used Structure version 2.3.3 (Pritchard *et al.* 2000; Falush *et al*. 2003) running for 100,000 iterations (including 10,000 burn-in), with the 'admixture' model and assuming independent allele frequencies. GIMLET software (Valière 2002) was used for pooling identical genotypes among several genotypes.

5. Results

In order to reach study objectives, an integrated data analysis approach was selected. Non-invasive genetic sampling (scat and forensic samples), camera-trapping, direct methods and interview results were matched to provide accurate data.

5.1. Genetic analysis

We have received 281 non-invasive biological samples: 198 swabs referring to livestock attacks and 88 scats. We successfully get genotypes from 73 swab samples (38.2%) and 32 scats (36.4%). PCR Amplification of all non-invasive samples was difficult to achieve, mainly from swabs samples. All samples produced incomplete genotypes although they were repeated until five times from two DNA extractions or until each allele was observed at least twice. In all samples, the average number of PCR amplifications per locus was 4.0 and an error value less than 10% from allele dropout or false allele amplification was detected. All the genotypes for 19 microsatellite loci are shown in the Annex.

Although we could not get complete genotyping for all 19 loci, we have proceeded with standard genetic analysis for 105 samples. Principal Coordinates Analysis (PCA) of all genotyped samples and dog and wolf reference genotypes showed a clear separation between the two reference sets. Swab and scat samples are distributed between the upper and bottom part of the plot, with some clearly sharing the wolf distribution area (Fig. 5).

After STRUCTURE analysis for species assignment, 51 of genotyped samples were assigned to the reference dog set and 46 samples were assigned to the reference wolf population (in red) (Fig. 6). From all samples, eight were not assigned to wolf, dog or wolf vs hybrid populations as they have shown very incomplete genotypes.

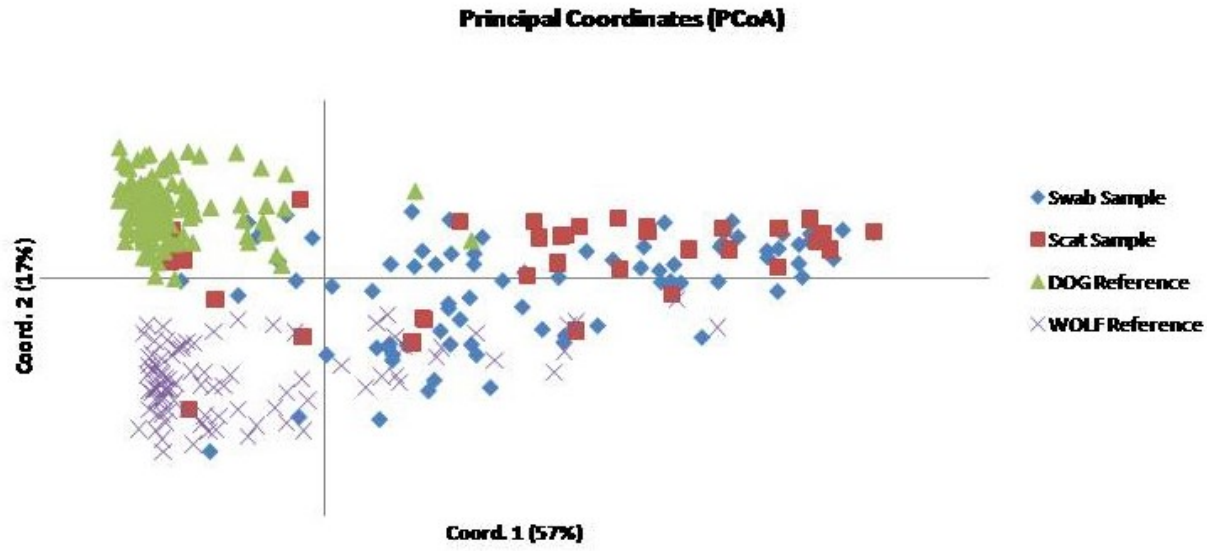

Figure 5 - PCA analysis of all amplified samples and two sets of genotype references: Dog and Wolf.

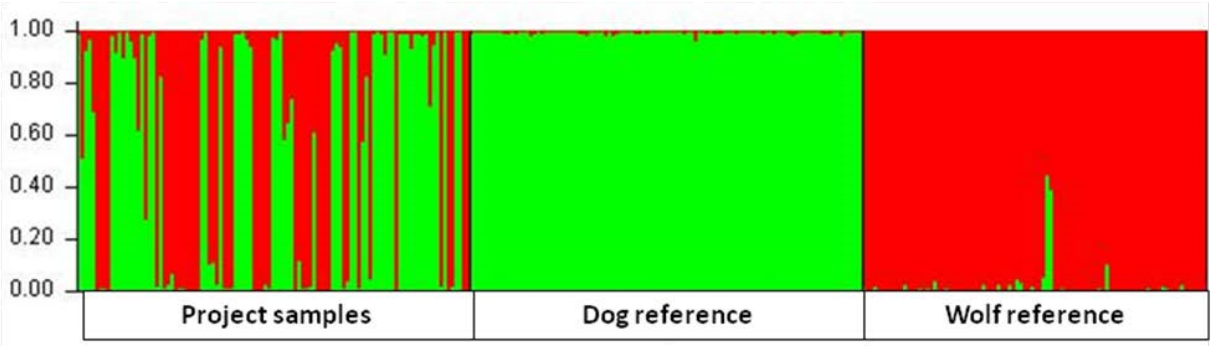

Figure 6 - Probabilistic assignment to the genetic clusters inferred by the Bayesian analysis with K = 2 of Dogs and Iberian Wolves reference genotypes.

For molecular sexing, we have selected the 46 samples assigned to wolf species by structure analysis. Molecular sexing revealed 39 males and 7 females as shown in Table 1.

Since all samples showed incomplete genotypes we analysed genotype matching using only 12 microsatellite loci. The cumulative probability-of-identity observed for those 12 loci was PID = 1.8×10^{-8}, or PIDsibs = 4×10^{-4}. The match probability (probability of one genotype to be produced a second time by chance = PID × population size) was PM=1.2×10^{-7},

considerably lower than PMsibs = $2{,}0x10^{-3}$ computed considering all the individuals to be related.

Table 1. Molecular sexing and species assignment for the 46 selected samples. (S refers to Swab samples and D to scats).

Project sample	Sexing result	Species assignment
SLIFE-17	MALE	Inconclusive
SLIFE-18	FEMALE	Wolf
SLIFE20	MALE	Wolf
SLIFE-21	MALE	Wolf
SLIFE23	MALE	Wolf
SLIFE-39	FEMALE	Wolf
SLIFE60	FEMALE	Wolf
SLIFE68	MALE	Wolf
SLIFE69	MALE	Wolf
SLIFE70	MALE	Wolf
SLIFE73	MALE	Wolf
SLIFE74	MALE	Wolf
SLIFE75	MALE	Wolf
SLIFE76	MALE	Wolf
SLIFE78	MALE	Wolf
SLIFE80	MALE	Wolf
SLIFE82	MALE	Wolf
SLIFE86	FEMALE	Wolf
SLIFE88	FEMALE	Wolf
SLIFE-89	MALE	Wolf
SLIFE-94	MALE	Wolf
SLIFE-96	FEMALE	Wolf
SLIFE-97	MALE	Wolf
SLIFE118	MALE	Wolf
SLIFE119	MALE	Wolf
SLIFE120	MALE	Wolf
SLIFE121	FEMALE	Wolf
SLIFE125	MALE	Wolf
SLIFE163	MALE	Wolf
SLIFE164	MALE	Wolf
SLIFE165	MALE	Wolf
SLIFE167	MALE	Wolf
SLIFE175	MALE	Wolf
SLIFE176	MALE	Wolf
SLIFE177	MALE	Wolf
SLIFE179	MALE	Wolf
SLIFE191	MALE	Wolf
SLIFE193	MALE	Wolf
DLIFE-4	MALE	Wolf
DLIFE-6	MALE	Wolf
DLIFE-11	MALE	Wolf
DLIFE-18A	MALE	Wolf
DLIFE-54	MALE	Wolf
DLIFE72	MALE	Wolf
DLIFE-74	MALE	Wolf
DLIFE78B	MALE	Wolf
DLIFE79	MALE	Wolf

Regrouping analysis using only the number of genotypes assigned to wolves (46), according to Gimlet regrouping algorithm and considering sexing analysis, produced a group of 32 distinct genotypes. The sex ratio calculated for the regrouped genotypes was 5.33 Male:1.00 Female.

5.2. Forensic genetic analysis

During the study period (January 2012 – October 2013), 52 canid attacks on livestock were forensically investigated out of 146 occurring in the study area. A total of 198 swabs (average 3.8/attack) and 4 hair samples were genetically analysed. In 31 attacks (59.6%) forensic analysis successfully confirmed wolf presence in 18 cases (35%), in 6 of which the presence of domestic dogs was also confirmed (Fig. 7).

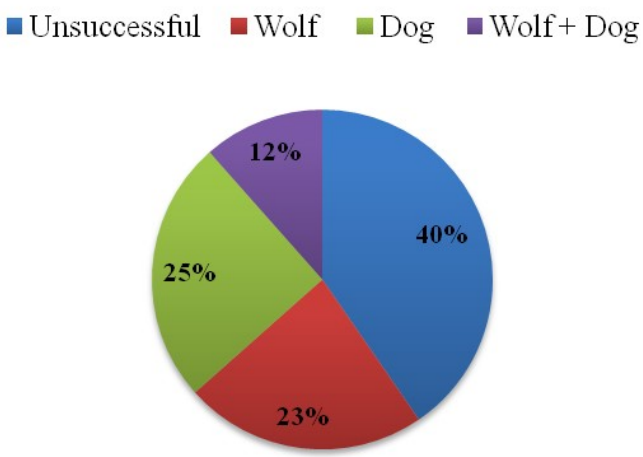

Figure 7 – Forensic genetic analysis of livestock damage events (N=52).

In 63.13% (n=125) of the 198 swab samples analysed it was no possible to extract DNA or had no sufficient quality to provide an accurate genetic species assignment (Fig. 8).

During forensic survey, wolves were identified in 37 swabs collected at canid damages on livestock and the presence of domestic dogs in 30 of them. Overall NGS survey (Fig. 9) produced 46 accurate wolf records.

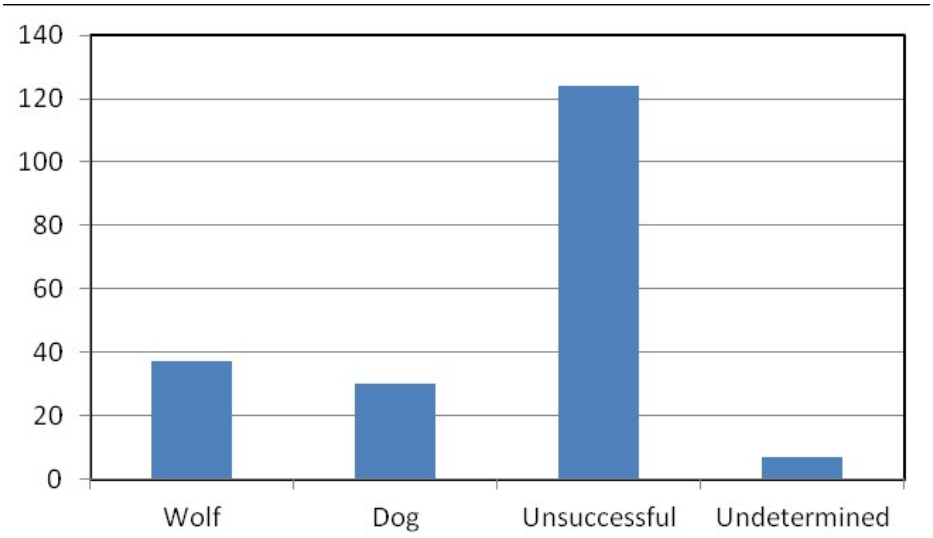

Figure 8 – Swab genetic analysis.

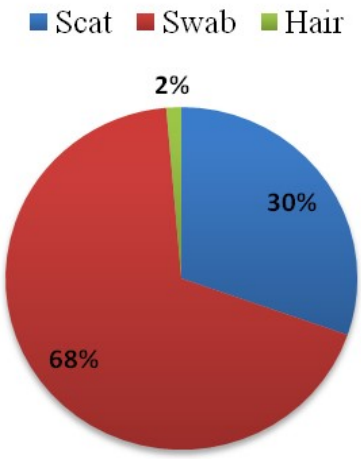

Figure 9 – Overall NGS effort (n=290).

5.3. Interviews to local people

Interview survey provided data of 49 wolf sightings during the study period. Positive interviews overlap extensively (80%) with the wolf presence data that were confirmed with the genetic results and camera trapping (Fig. 10).

The results indicate potential wolf presence in areas where it was not detected (8 UTM) with genetic analysis or camera trapping, which must be validated (or not) in future works.

No reliable information was obtained relating to wolf mortality or breeding records.

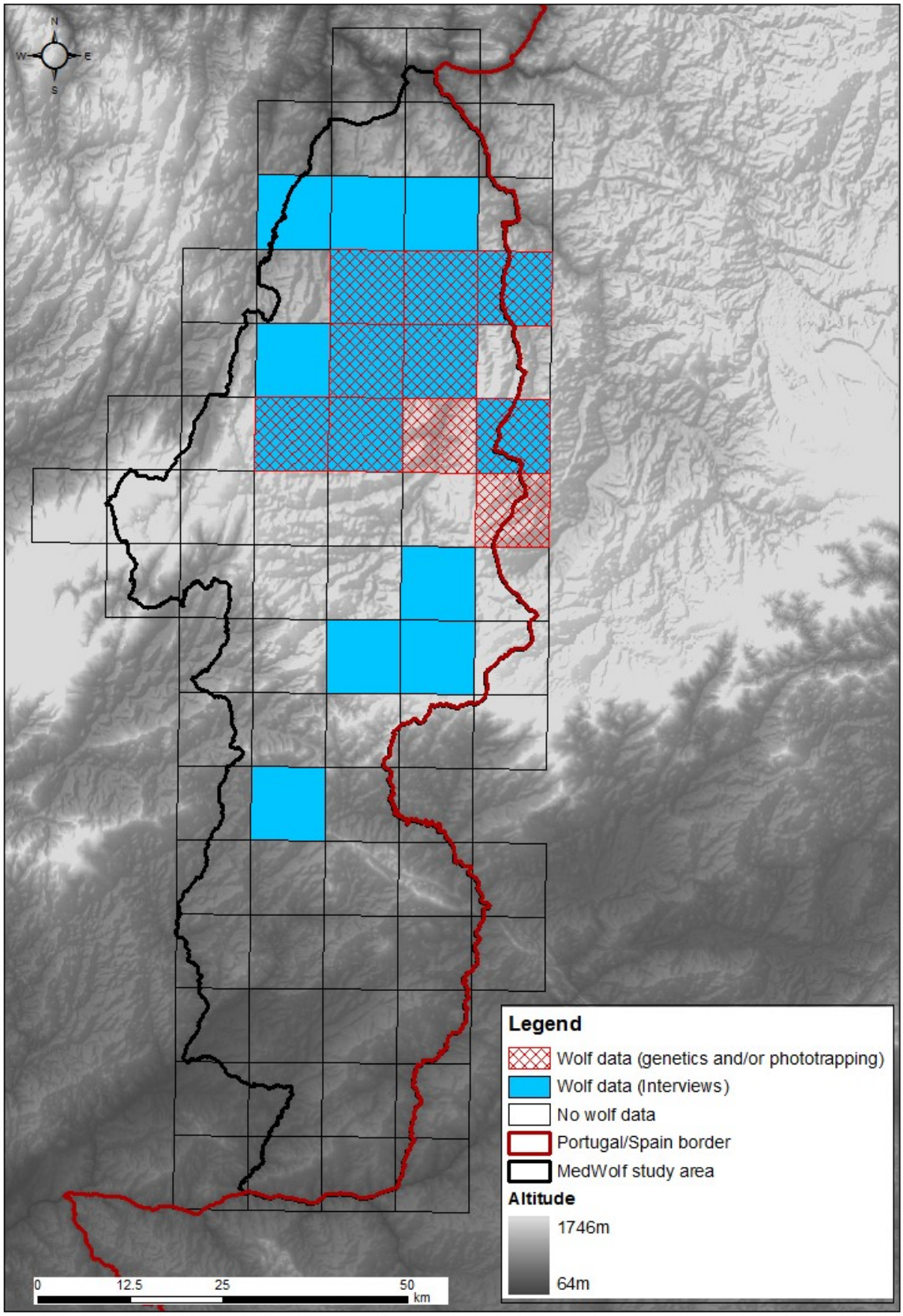

Figure 10 - Interview survey data and confirmed wolf presence in the study area considering genetic analysis (scats and swabs) and camera trapping.

5.4. Wolf sign survey

A total of 88 scats (Figs. 11, 12) were collected and genetic analysed. It was possible to accurately assign species in 32 samples (36.4%): 23 were identified as dog and 9 as wolf.

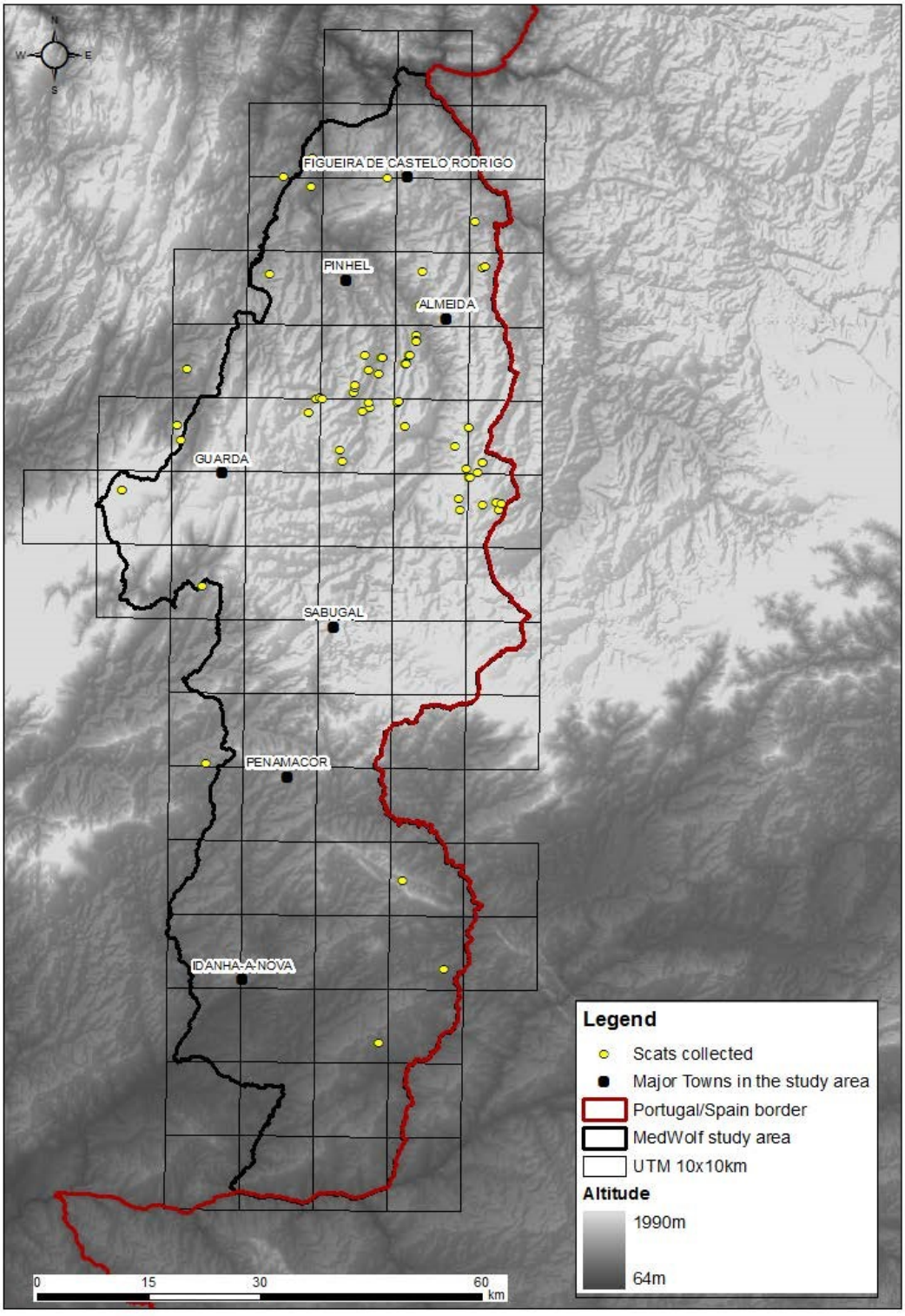

Figure 11 – Spatial distribution of scats collected in the field and send to genetic analysis.

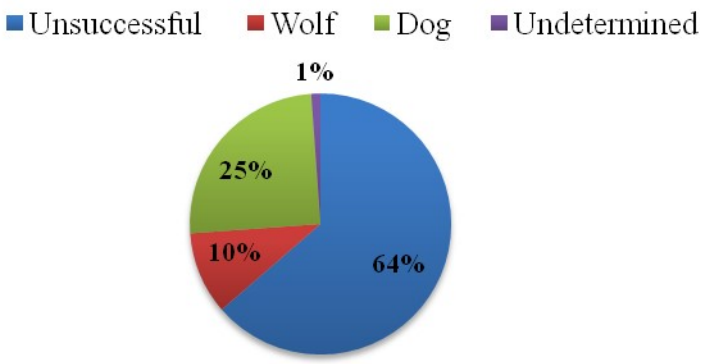

Figure 12 – Scat genetic analysis (N=88).

Wolf scat were collected only in 6 UTM out of the 65 squares surveyed (9.2% of the study area). Kilometric Abundance Analysis (KAI) was not calculated due to the small sample size and its spatial distribution, where most of the squares had only one wolf scat confirmed and the resulting KAI would be very close to zero.

5.5. Camera trapping and photograph records

Camera trapping (Figs. 10, 13) produced 25 wolf pictures (in 4 different UTM) in 6 events (frequency of capture = 1%).

Figure 13 – Adult wolf caught on a baited camera-trap.

All pictures portrayed a single adult wolf. Only in 1 of these 4 locations were obtained, separately, wolf and dog photographs. A single adult wolf photograph was taken by a local resident using a regular camera.

5.6. Direct methods

No wolf positive results were obtained. During wolf howling sessions only domestic dog barking responses, from nearest villages or farms, were obtained.

5.7. Mortality

No data was obtained, during field work and from the official records, on wolf mortality.

5.8. Scat detection dog team

Scat detection dog team performed four short wolf scat surveys in the last two study months. In these sessions, 5 samples were positively alerted (Fig. 14): 1 genetically confirmed and 4 with inconclusive genetic results.

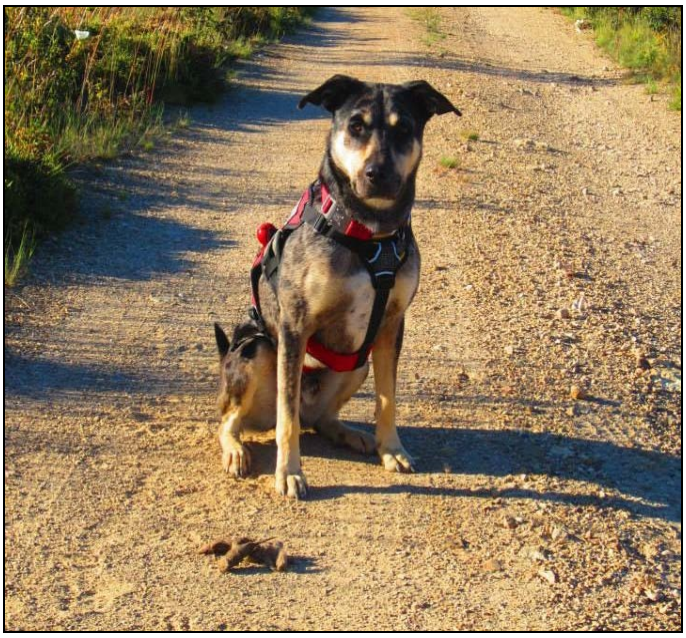

Figure 14 - Wolf scat detection dog Zeus sample alert.

Dog team positively alerted scat “86” sample site (genetically confirmed) was selected to set a camera-trap with subsequent positive results (Fig. 15).

Figure 15 - Wolf photograph on positively alerted scat 86 site.

5.9. Official damage reports

Damage reports in the study area varied significantly across the study area, with Almeida municipality reporting the higher number of wolf attacks (n=81), although Guarda and Sabugal had similar number of animals attacked (n≈165) despite smallest number of attacks, while Penamacor and Idanha-a-Nova, the southern municipalities, reported no wolf damage (Table 3).

Table 3 – Official data on wolf damage in the municipalities of the study area.

Municipality	Nr. attacks	Nr. animals killed	Nr. animals injured	Nr. animals disappeared	Total
Figueira de Castelo Rodrigo	1	8	0	0	8
Pinhel	9	23	1	4	28
Almeida	81	107	44	14	165
Guarda	22	60	13	92	165
Sabugal	20	74	50	44	168
Penamacor	0	0	0	0	0
Idanha-a-Nova	0	0	0	0	0
Total	**133**	272	108	154	534

The following figure (Fig. 16) show the distribution of the reported wolf attacks to domestic animals, in the parishes of the study area.

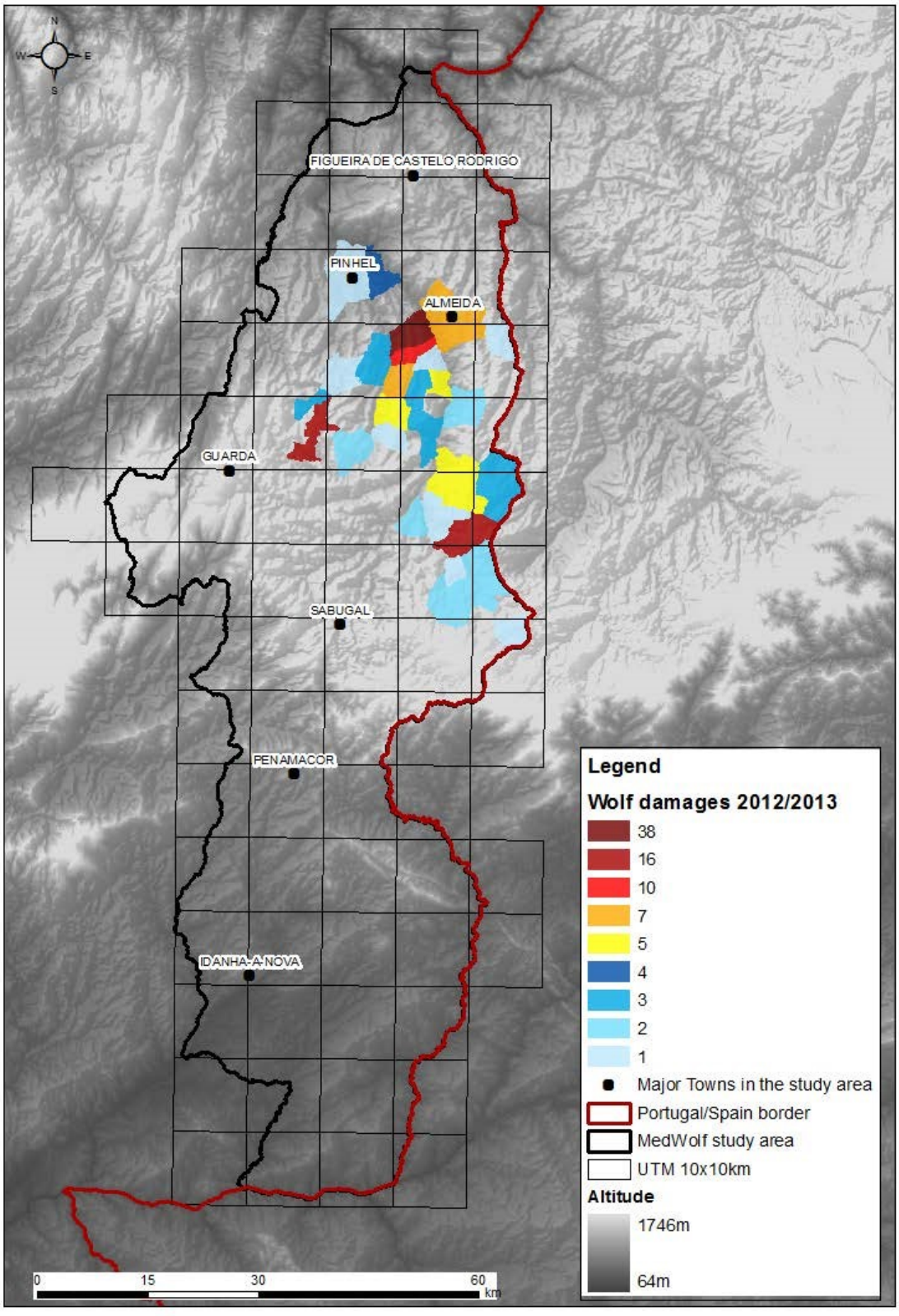

Figure 16 – Distribution of reported damage (and accepted by ICNF) per parish, in the study area.

5.10. Wolf distribution

Despite different sampling effort from different methods, wolf survey (NGS and camera trapping) results were able to confirm wolf presence in 10 UTM squares (19.8% of the study area) (Fig. 17).

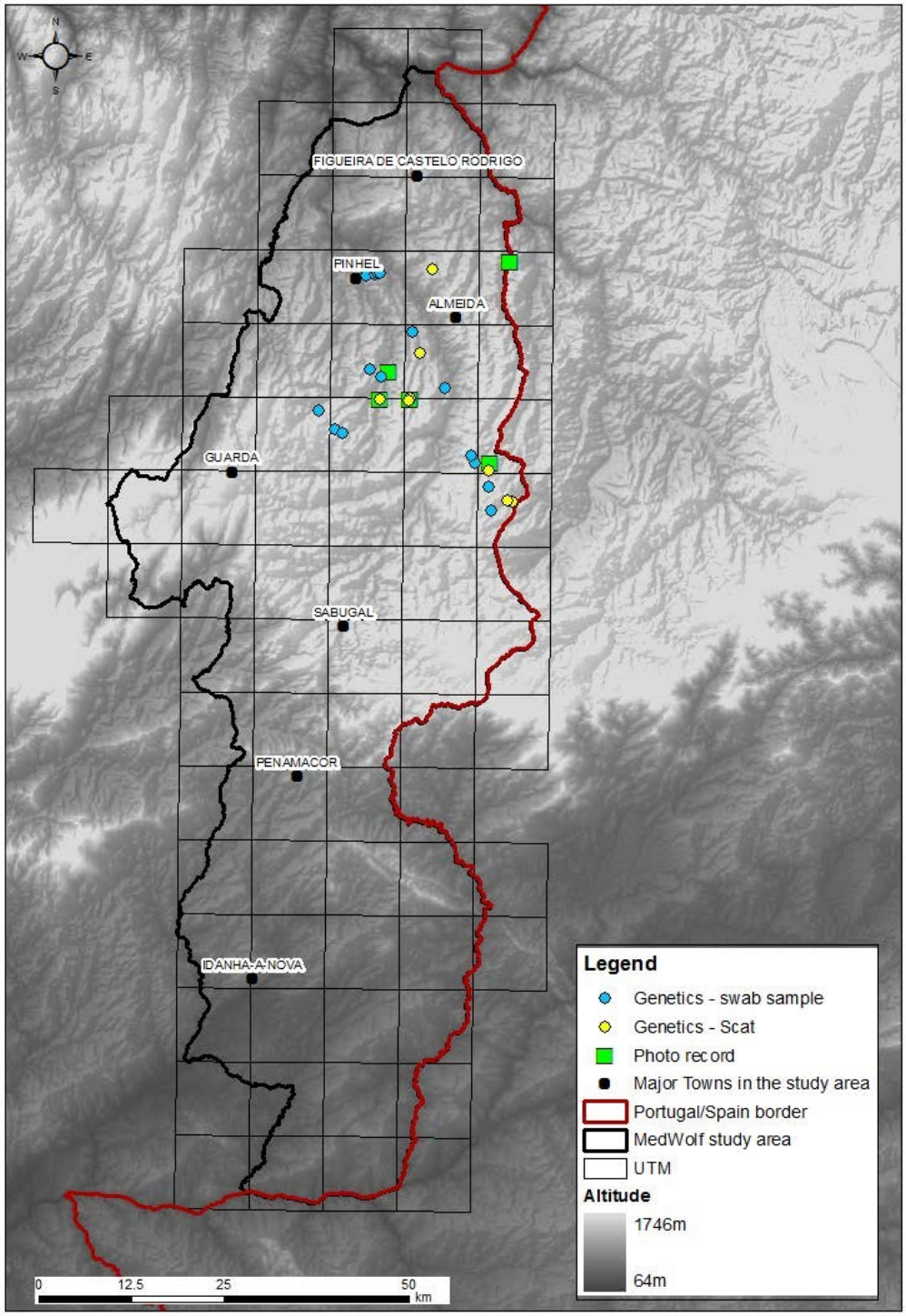

Figure 17 – Wolf confirmed presence (NGS and photograph records) in the study area.

Wolf range analysis shows 4 occupied areas: a) Northeast Almeida (Malpartida/Vermiosa); b) South of Vilar Formoso (Malhada Sorda, Nave de Haver, Aldeia da Ribeira); c) West Almeida/Cabras' Creek (Freixo, Azinhal, Atalaia, Lamegal) and Pínzio (Castanheira, São Pedro do Jarmelo, Ribeira dos Carinhos, Gagos).

Forensic analysis was the most effective method for confirming wolf presence (n=18) (Fig. 18). Camera trapping provided 6 records and genetic analysis of scats, 9 records. NGS genetic analysis shows a strongly overlapped wolf and free-ranging domestic dog distributions in the study area (Figs. 19, 20).

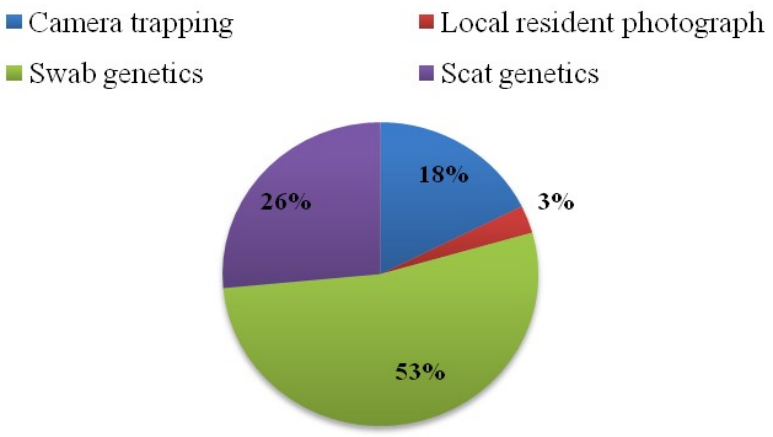

Figure 18 – Wolf presence confirmation (n=34) using non-invasive methods.

Figure 19 – Free-ranging dogs feeding on donkey carcass.

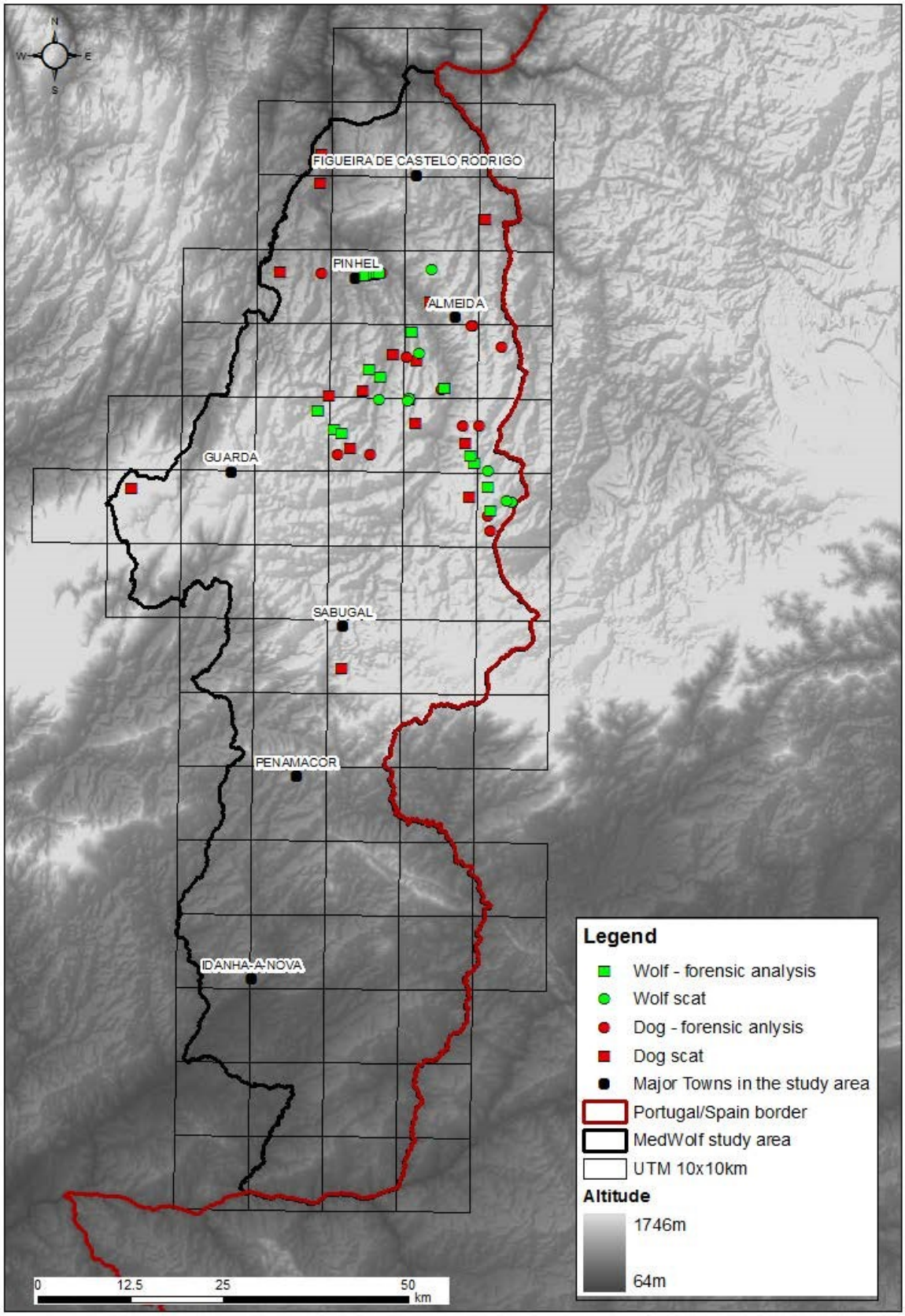

Figure 20 - Wolf and free-ranging dog confirmed presence based on scat and swab samples genetically analysed.

Wolf and domestic dog damage on livestock can occur in close vicinity of each other. The same happens to the frequent presence of dogs in potential wolf habitat (as confirmed by scat samples). Wolves and free-ranging dogs are sympatrically distributed throughout the entire wolf range in the study area.

5.11. Wolf reproduction

No data could confirm or suggest wolf reproduction in 2013. Only one interview record suggested wolf breeding in 2012 - the observation of 4 different sized wolves (possibly 2 adults and 2 juveniles), west of Almeida municipality, in October 2012.

5.12. Wolf packs and population estimates

Only the official damage reports confirmed the presence of the wolf packs throughout the estimated wolf range. No results obtained with direct methods could attest wolf presence.

Wolf pack A (*Confirmed*) – This pack was firstly confirmed in 2012 (Cadete *et al.* 2012) through a non-invasive wolf survey (NGS and camera-trapping) in the region. In pack A potential home range, a total of 31 attacks (3.1 attacks/month) on livestock were registered from January to October 2013. Sightings in its core area were very common (n=20) and 9 interviewees described reliable sightings of 2 wolves together during the winter of 2013. A local hunting ranger reported a sighting of 4 wolves in October 2012 and 2 wolves were photographed by camera-traps during an attack to an ostrich farm in the same year. This pack's home range is located north of the highway A25 (west of the Côa River) and its core is included in the Cabras's Creek area (Fig. 21). Its northern limit is in the region of Pinhel town.

Wolf pack B (*Unconfirmed*) – This pack (known as Jarmelo pack) was classified as "probable" in the 2002-03 national wolf survey (Pimenta *et al.* 2005). The analysis of reported livestock damage (average 1.3 attacks/month) was only enough to classify this pack as "unconfirmed" although there were reliable interview records about two sightings of two wolves travelling together in its possible home range. Pack B territory is crossed by a main highway (A25) that presumably is not an ecological barrier for wolves, due to large wildlife permeable underpasses. Due to wolf mobility and ecology it is possible that pack B (identified in the national wolf survey) could be the same as pack A, that moved slightly East adapting to landscape and food availability shifts.

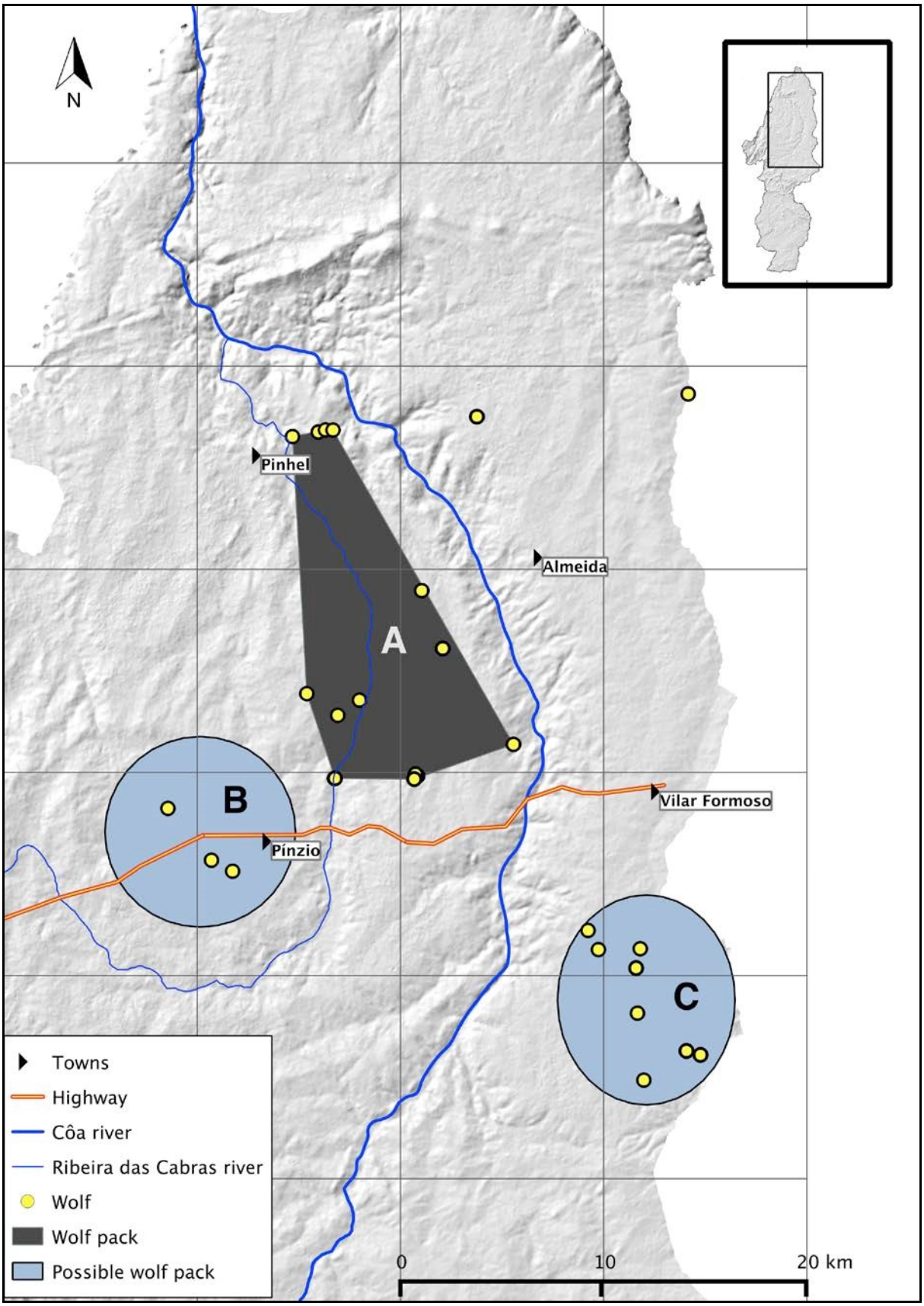

Figure 21 - Wolf packs' location.

Wolf pack C (*Unconfirmed*) – This pack (known as Sabugal pack) was classified as "probable" in the Portuguese wolf census (Pimenta *et al.* 2005). During 2013 there were no interview records of more than one individual wolf sightings, but considering the NGS results and the analysis of official damage data (average 1.2 attacks/month) this pack is classified as "unconfirmed". Camera trapping was unsuccessful, although there was a photograph record of an adult wolf obtained by a local resident (Fig. 22). Pack C probable home range, which may extend to Spain, covers the southern part of the Almeida and Northern Sabugal municipalities. Aldeia da Ponte area is likely to be the southern limit of this pack probable home range.

Figure 22 – Adult wolf photographed by a local resident in the probable home range of pack C.

In the extreme north-eastern part (Malpartida/Vermiosa) of the wolf range in the study area, camera trapping results (4 photographs) revealed the presence of at least one adult wolf (all photos showing single indistinguishable animals). Although different data (NGS) (Cadete *et al*. unpubl. data) and interview results suggested the past existence of a pack (2011/2012), during the study period only one reliable interviewee described the sighting of two wolves. Therefore, it was assumed that in this border region at least one solitary wolf (SW) (Fig. 23) was identified during the current study. The distance from the potential packs A, B and C makes it less probable that this animal belongs to those packs.

Figure 23 - Detected probable solitary wolf.

Population nucleus size was estimated (Table 4) considering two time periods (before and after the breeding season), five case scenarios and population estimates from Portuguese last wolf census for southern Douro areas (Pimenta *et al*., 2005):

1. Confirmed pack A (CWP A) was the only pack occurring in the study area;
2. Confirmed pack A was the only pack occurring in the study area and its home range included wolf range classified as pack B. A and B home range hypothesis is based on their close proximity and high connectivity potential as well as its calculated size (Minimum Convex Polygon MCP AB = 195 km^2);
3. Pack A and pack B were the only occurring packs in the study area;
4. Pack A and pack C were the only occurring packs in the study area;
5. Packs A, B and C occurred simultaneously in the study area.

As described in last Portuguese wolf census report, population size estimate was calculated assuming the presence of one SW and the probable existence of non-detected solitary or dispersal wolves (NSDW) in the area.

Table 4 - Population size and average population size estimates considering five case scenarios. **CWP** (Confirmed Wolf Pack); **UWP** (Unconfirmed Wolf Pack); **SW** (Solitary wolf); **NSDW** (non-detected solitary and/or dispersal wolves).

	Population size		**Average population size**
	DEC 12 – APR 13	MAY 13- SEP 13	DEC 12 – SEP 13
1 CWP A + SW + NSDW	3 + NSDW	8 + NSDW	5.5 + NSDW
1 CWP AB + SW + NSDW	3 + NSDW	8 + NSDW	5.5 + NSDW
1 CWP A + UWP B + SW + NSDW	5 + NSDW	15 + NSDW	10 + NSDW
1 CWP A + UWP C + SW + NSDW	5 + NSDW	15 + NSDW	10 + NSDW
1 CWP A + UWP B + UWP C + SW + NSDW	7 + NSDW	22 + NSDW	14.5 + NSDW

Considering MCP for the entire wolf range using all confirmed wolf signs (scats, damages and photos) we would have a wolf range of 645 km^2 within our study area and an average wolf density ranging from 0.8 wolves/100 km^2 to 2.2 wolves/100 km^2.

Considering wolf range as the total presence confirmed in UTM squares (10x100 km^2 = 1,000 km^2), average wolf density ranged from 0.6 wolves/100 km^2 to 1.4 wolves/100 km^2.

5.13. Population nucleus trend

Data suggest a wolf range increasing trend from 2002-3 to 2013 (Fig. 24), although the Portuguese wolf census (Pimenta *et al.* 2005) did not include NGS and camera trapping methods. Despite some differences in methodologies, the effort made with the traditional sign survey was the same from 2002 to 2013, since the whole UTM 10x10 km square in the study area were surveyed and was this method that gave us most of the wolf data, in conjunction with forensic data gathered from wolf damages (that can also occur throughout the study area). Camera trapping was used opportunistically, and despite some good results, it only added one square to the UTMs where wolf presence was confirmed with other methods.

Nevertheless, the increasing number of presence confirmed UTM squares indicates a probable "stable to increasing" wolf range in the region. Recent wolf presence confirmed squares indicate a north oriented nucleus expansion.

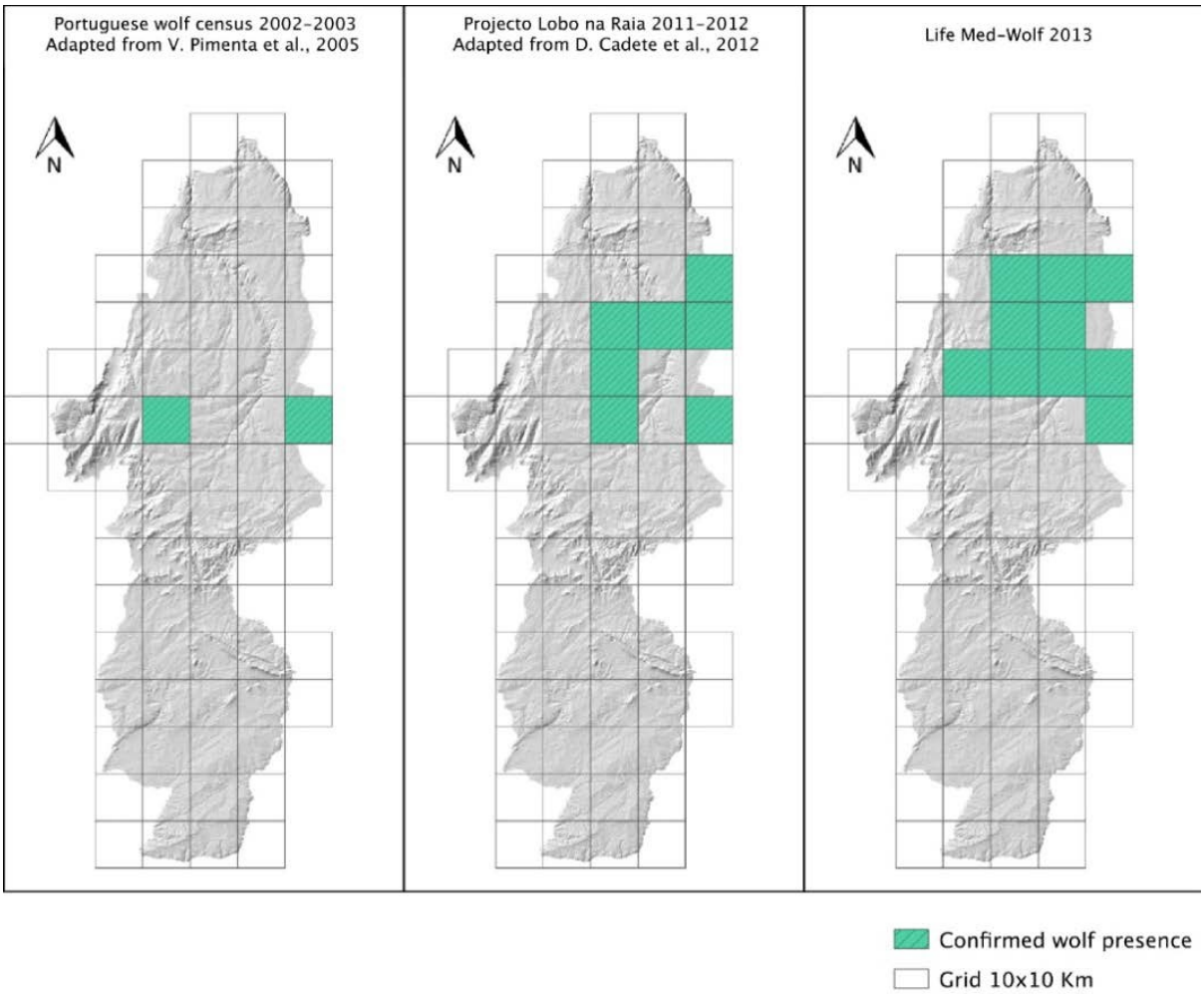

Figure 24 - Wolf population nucleus trend from 2002 to 2013 in the study area, as confirmed by wolf signs survey, complemented in 2011-2012 and 2013 with opportunistic camera trapping.

6. Discussion

6.1. Molecular data analysis

Genotyping from swab samples (38.2%) and scats samples (36.4%) was slightly lower when compared with other studies previously performed by the same team, mostly on the wolf population North of the Douro River (>45% for scats, Borges *et al.* 2012, and around 42% for swabs, Borges *et al.* 2012a).

Concerning swab samples, the unknown time between the livestock attack and sample collection may be an important reason for failure. Some swabs did not show any sign of blood or body fluids in the cotton part. Amplification result was completely null and contributed to a lower success percentage. The sample collection method is now under review to try to

introduce technical improvements (*e.g*. to ensure swab sawing in case of dried damage samples).

For scats, the amplification success was lower when compared to swabs. One of main reason, although the study area exhibit mostly a dry soil, is that cold nights and frost, followed by mild temperatures during the day often contribute to the lyse of epithelial cells that are the biological source of DNA.

For both type of samples no complete genotypes were obtained. However, wolf species assignment was achieved for 46 samples. Those 46 genotypes, being incomplete, made difficult the identification of exactly matching genotypes. A regrouping test using GIMLET software (Valière *et al.* 2002) produced 32 possible individual genotypes after matching. This number of individuals seems still high considering Grupo Lobo knowledge about the studied area. Overestimation of the number of individuals can be due to genotyping errors as allelic dropout or false allele amplification (Broquet & Petit 2004, Pompanon *et al.* 2005) that were not minimized with the repetition of PCR amplification of microsatellite loci. Degradation of target DNA is the most frequent reason for this.

During the month of January 2014, we still try to get complete genotypes (even trying new forensic loci) for those 32 genotypes. Until the end of March 2014, we will expect to get more loci in order to achieve some complete genotypes and confirm the best candidates for genotyping match as wolf monitoring in the study area will be resumed in action D.3 of the project.

Briefly, using molecular techniques we have detected the presence of wolves in the study area from swabs and scat samples, confirming the hypothesis of a re-colonization event. DNA quality has limited the chance of genotyping for all loci. Although we reached a final number of 32 individuals, we think that this number is overestimated (Pompanon *et al.* 2005). This is a very common conclusion when using molecular methods for estimating the number of individuals due to genotype errors resulting from forensic and NGS samples analysis. After the molecular analysis of all samples collected (about three times the expected number in Action A.2) the success rate on genotyping was below what we have obtained in similar studies from the North of Douro's River (Borges *et al.*, 2012). Age of samples (scats) in the field, time between damage event, damage alert and sample collection time may be crucial for good genotyping rates.

We think that the results obtained in Action A2 must be considered as a baseline study from which subsequent actions, namely D3, will benefit. This will include the improvement of sample collection protocols (preventing any subsequent DNA degradation) and the

selection of a minimum number of loci showing lower genotyping errors and maintaining high values of Probability of Identity. We believe that this strategy will improve genotyping success and a more reliable individual identification and population estimation.

6.2. Wolf monitoring and survey challenges

Wolf monitoring in the study area carried some challenges. Several landscape, ecological and social features made wolf survey design and implementation a difficult task:

a) Sign survey conditioned in private large fenced natural or semi-natural areas that included potential wolf habitat

b) Abundance of free-ranging dogs

c) Abundance of vultures and other scavengers can bias forensic analysis;

d) Due to the past near absence status, wolf knowledge and presence awareness was lost and many wolf damage on livestock and/or wolf sightings were probably unreported or unreliable. Additionally, the increased wolf conflict in the last years (mainly related with livestock losses) hampered day to day survey actions, making the collection of data more difficult to obtain. Trans-boundary wolf home ranges are also difficult to survey as it implies Spanish-Portuguese institutional cooperation and foreign interviewing.

6.3. Non-invasive genetic sampling and forensic analysis

Non-invasive genetic sampling (Echegaray & Vilá 2009, Galaverni *et al.* 2012, Marucco *et al.* 2009, Marucco *et al.* 2012, Roque *et al.* 2011) is a useful tool for accurately survey elusive fauna and in particular, the wolf. NGS capture-recapture methods (Marucco *et al.* 2009) can address reliable wolf survival and population trend, although their application requires great effort. In the current study, non-invasive genetic scat and swab sampling survey was fundamental to achieve a minimum viable insight to wolf presence and distribution in the study area. Swab sampling showed greater success rates than scat analysis in species presence confirmation. Dog and red fox abundance, local climate conditions, sample exposure time in the field and other sample degradation factors could have affected sample identification and DNA extraction rates. Overall results showed the importance of non-invasive genetic sampling to estimate minimum wolf range, being camera trapping and direct methods less efficient. In fact, an effort was made to increase sample size for genetic analysis, by collecting swabs from the maximum number of damage sites possible (35% of the damages occurring during the study period, resulting in a total of 198 swabs), since the number of scats collected

in survey transects in low density populations is usually low (88 scats in this study in 310 km prospected). Despite this effort, genotyping matching for individual identification was not possible due to low DNA quality. DNA degradation on the field seems to be the most important factor to take into consideration when applying molecular techniques on more fine analysis, such as genotype matching for individual identification and relationship estimates. Therefore, genetic analysis was only used for species assignment and was not used to estimate minimum population size (capture-recapture), pack home ranges and breeding occurrence to avoid biased results during this action.

Forensic analysis achieved good species assignment rates, although climate and carcass exposure time could have decreased success rates. Wolf and dog presence detection rates on livestock damages were similar, although wolf detection was slightly higher. Simultaneous wolf and dog detection could have been related with dog scavenging behaviour on wolf kills. Dog presence data on livestock damage should be carefully analysed in order to avoid scavenging behaviour and swab sampling bias. Nevertheless, data analysis indicated probable non-wolf related livestock damages caused by uncontrolled/free-ranging domestic dogs.

6.4. Wolf distribution, packs and population nucleus status

Although capture-recapture methods could not be applied, non-invasive genetic sampling and camera trapping results showed a potential "stable to increasing" wolf population nucleus trend, especially when compared with the last Portuguese wolf survey from 2002/2003 (Pimenta *et al.* 2005). Since 2011-2012, confirmed wolf range has slightly increased, but results could have been biased by increased survey effort. Reliable interview results showed potential wolf presence areas that were not identified through NGS, camera trapping and direct methods. These areas should be surveyed carefully in future monitoring actions. One confirmed pack had already been previously detected (Cadete *et al.* 2012) as well as other two unconfirmed packs (Sabugal and Jarmelo) (Pimenta *et al.* 2005). Proximity and habitat connectivity potential between confirmed pack A and unconfirmed pack B (Jarmelo) should be taken into account in future surveys. The data seems to support the possibility of existing only one confirmed pack in the western area of the estimated wolf range. Large scale wolf distribution analysis confirmed previous studies results (Pimenta *et al.* 2005, Cadete *et al.* 2012): stable wolf presence was detected in a small part of the study area (north-eastern section).

6.5. Threats and wolf conservation

Although no mortality and/or poaching data were collected, several factors suggest high chances of their occurrence. Non-detected reproduction and slow wolf range growth rates suggest non-reported mortality and poaching events. Illegal wild boar snaring is popular throughout the study area as well sport hunting with little surveillance. Carcass poisoning and "sit and wait" hunting actions are expected to increase if livestock damage increases. Domestic dog abundance and generalised wolf - dog sympatry throughout the wolf range can be a major threat for wolf recovery in the region. Free-ranging dog damage on livestock can contribute to an increasing wolf-human conflict and wolf persecution. Although no wolf-dog hybrid or "wolf and dog" packs were detected, wolf and free-ranging dog overlapped distribution can constitute an important threat for wolf conservation in the region.

6.6. Wolf scat detection dog team

Scat detection dogs have been used in the last decade to increase accuracy and biological sample size in different target-species studies, including large carnivore ecological, genetic and physiological research (Wasser *et al.* 2004). Studies comparing different study methods (Long *et al.* 2006) showed the efficiency and reliability of this non-invasive technique. In this present study, despite its reduced use, the data obtained with the dog team show the great potential of this method in the study area. The conditions of the study area - low wolf density, abundance of free-ranging dogs and red foxes - and the need to increase sample size and sample quality (fresh or non-degraded scats) recommend the use of this method in the future.

7. Final Considerations and Recommendations

Despite the monitoring challenges, that determined the use of a combined monitoring program, the overall study goal was achieved. The entire study area was monitored using transects for scat recognition and wolf damage data was collected throughout the study area, wherever they occurred. Genetic analysis was used to validate the field data, and camera trapping was used only in areas with some kind of wolf information (from field data or interviews) to confirm and visually register wolf presence. Camera trapping was used mainly in the northern part of the study area, due to limited material and due to the concentration of wolf information in that area. For this reason, camera trapping alone did not give us much more additional information on wolf presence than the others methods (enabled the

confirmation of wolf presence on only one UTM 10x10 where there was no other information), thus it did not biased the overall survey effort.

As a result we can say that a reliable wolf distribution map was produced showing a potential "stable to increasing" population nucleus trend that occupies only a small part of the surveyed area. Forensic analysis provided reliable data that attested wolf and domestic dog presence in several livestock damage events. Wolf / dog related livestock losses and wolf-dog sympatric distribution can increase wolf - human conflicts and threaten wolf recovery in the region. Non-invasive methods showed good results although effort should be done to improve scat sample size and quality. The use of the scat detection dog will surely suit this need. Damage prevention, public awareness and anti-poaching surveillance continued actions are required. Conservation efforts should target connectivity improvement of these packs with the Spanish (east) and the Portuguese (north-western south of the Douro River) wolf nuclei. Gene flow research should be run to address border nucleus, available corridors and migration patterns. Habitat suitability, ecological connectivity and wolf monitoring research should be developed to address wolf absence causes in International Douro Natural Park and Malcata Mountain Natural Reserve. It is also recommended the implementation of wolf feeding ecology and wild prey availability studies.

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