2nd International Workshop on the genetic study of the Alps – Dinara - Pindos and Carpathian brown bear (*Ursus arctos*) populations

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2nd International Workshop on the genetic study of the Alps-Dinara-Pindos and Carpathian brown bear (*Ursus arctos*) populations

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1. INTRODUCTION

1.1. "Why genetics?"

In the past 30 years molecular techniques have evolved rapidly and led to an increased interest of their application in the conservation and management of wildlife. Recently, non-invasive sampling in combination with genetic research have proven especially efficient in the study of endangered species; within this group of animals brown bears (*Ursus arctos*) have served as a model species for the application of these methodologies.

Despite considerable conservation efforts throughout the world and Europe in particular, the status of brown bear populations remains in some cases unknown, while in other, populations continue to decline. One of the main reasons for this dire situation is the nature of the species itself. Bears are shy and elusive animals with large home ranges that require large patches of well-connected habitat that can span over several hundreds of square kilometers and across national borders. This makes brown bear research, management and conservation actions much more effective if they are carried out on a larger, multinational scale. However, the legislative, logistic and scientific differences between the various countries involved make this task very challenging. Almost nowhere in the world is this more evident than in the Alps – Dinara - Pindos brown bear population that spans over ten different countries (i.e. Austria, Italy, Slovenia, Croatia, Bosnia & Herzegovina, Montenegro, Serbia, The Former Yugoslav Republic of Macedonia, Albania and Greece). If one includes the brown bear populations in the Carpathian and Rila – Rodopi mountains, which would be required for a comprehensive understanding of the status of the species in the region, the number of countries, with the addition of Slovakia, Romania and Bulgaria, rises to 13. It is evident therefore that effective research, conservation and management of brown bears in the region can be achieved only if all the involved stakeholders in the various countries are brought under a common framework.

The need for a common strategy in managing the fate of *Ursus arctos* in the region, at least on a genetic level, was acknowledged already early on and led to the 1st International Workshop on the genetic study of brown bears (*Ursus arctos*) of the Alps – Dinara - Pindos and Carpathian populations in 2007.

1.2. "1st International Workshop on the genetic study of the Alps – Dinara - Pindos and Carpathian brown bear (*Ursus arctos*) populations"

The workshop was held at the Jelen Sneznik Hunting Reserve in Masun, Slovenia on the 1st & 2nd October 2007 and was attended by twelve specialists from four different countries. The workshop focused mainly on the status of genetic research carried out at the northern range of the Alps – Dinara - Pindos bear population and identified one outstanding conservation / research priority. This was to create a network of collaborating laboratories in order to develop:

1) a standard protocol for genetic identification and,
2) a common genetic database for brown bear populations in the Alpine - Dinaric region.

The aim of this partnership would be to:

1) identify the minimum number of microsatellite loci required to standardize individual genotypes and,
2) improve national / laboratory protocols and procedures.

The workshop came to the following conclusions:

1. Collaborating laboratories in the region should use a common panel of microsatellites and a common research and laboratory procedure protocol.
2. A common system for allele calling should be determined.
3. All samples from the collaborating laboratories should be analyzed using the common protocol and the data exchanged.
4. Collaborating laboratories should meet again after the proposed tasks are completed in order to plan future research activities.

Following this first meeting, participating countries / laboratories went on to pursue the conservation and research priorities identified. However, the ever-growing general interest in the genetic research of the species in the region in combination with the production of new data on the genetic status of brown bears in other countries such as Greece and F.Y.R. Macedonia in the southern part of the Alps –

Dinara - Pindos population and Slovakia, in the Carpathian population, led to the organization of a second workshop in 2009.

1.3. "2nd International Workshop on the genetic study of the Alps – Dinara - Pindos and Carpathian brown bear (*Ursus arctos*) populations"

The 2nd International Workshop on the genetic study of brown bears (*Ursus arctos*) of the Alps – Dinara - Pindos and Carpathian populations was held on a sunny weekend of May 2009 at the facilities of the Veterinary Faculty of Zagreb/Croatia. The workshop was organized by Magda Sindičić and Prof. Đuro Huber and was attended by 20 participants from five different countries (Croatia, F.Y.R. Macedonia, Greece, Italy and Slovenia). In addition, another 20 conservationists and scientists from five more countries participated in the pre- and post-workshop activities (i.e. preparation of the workshop agenda, preparation of a workshop report). This workshop is considered a follow-up of the 1st workshop held in Masun / Slovenia in 2007 and its main priorities were to pursue the conservation and research aims already identified, while integrating in this process additional countries that are either currently carrying out genetic research, such as Greece and Slovakia, or are considering initiating their own genetic monitoring efforts, such as F.Y.R. Macedonia and Serbia.

Discussions during the two-day workshop were under the (strict) time coordination of Prof. Đ. Huber, while T. Skrbinšek was responsible for keeping notes of the discussion. In the first day of the meeting the current status of genetic research in the region was presented – seven PowerPoint presentations from six different countries (Italy presented two different projects) were held. The information presented during the workshop is summarized in Table 1 and presented in more detailed form as project fact sheets in Annex D.

Table 1: Main characteristics and results of the seven genetic projects on brown bears in the Alps – Dinara - Pindos and Carpathian Mountains regions

Country	Study area	Study design	Sample type	Use of bait	Loci screened	Loci used for sex identification	Nr. of individuals identified
Austria	The Alps of Austria	Opportunistic & systematic sample collection	Scat & Hair	YES	G1D, G10B, G10L, G10P, Mu23, Mu26, Mu50, Mu59	SRY, Amelogenin	22
Croatia	Dinara Mountain range in Croatia	Opportunistic & systematic sample collection	Tissue & Scat	NO	G1D, G10B, G10C, G10J, G10L, G10M, G10P, G10X, Mu10, Mu23, Mu50, Mu51, Mu59,	SRY	354 (144 tissue samples of hunted bears and 210 scats)
Greece	Northern Pindos mountains in Greece, south-western Albania, eastern F.Y.R. Macedonia, Serbia	Opportunistic & systematic sample collection	Tissue & Hair	NO	G1A, G1D, G10B, G10C, G10H, G10J, G10L, G10M, G10P, G10O, G10U, G10X, CXX20, CXX110, Mu23, Mu26, Mu50, Mu51, Mu59, Msut-2, REN145P07	SRY, Amelogenin	198
Italy - 1	Central Alps: Trentino	Opportunistic & systematic sample collection	Scat & Hair	YES	G1A, G1D, G10B, G10C, G10H, G10J, G10L, G10M, G10O, G10P, G10X, Mu05, Mu10, Mu11, Mu15, Mu23, Mu50, Mu51, Mu59, CXX20	Amelogenin	43
Italy - 2	Eastern Alps: Friuli Venezia Giulia	Systematic sample collection	Hair	YES	G1D, G10C, G10L, G10M, G10P, Mu10, Mu15, Mu23, Mu50, Mu51, Mu59	Amelogenin	10
Slovakia & Romania	Western & Eastern Carpathians in Slovakia and Romania	Opportunistic & systematic sample collection	Tissue & blood & bone & scat & hair	NO	G10B, G10C, G10D, G10J, G10L, G10M, G10P, G10X, Mu10, Mu23, Mu50, Mu51, Mu59	SRY	373
Slovenia	Dinara mountains in Slovenia, Slovenian Alps	Systematic sample collection	Scat & tissue & Hair	NO	G1A, G10B, G10C, G10D, G10H, G10J, G10L, G10M, G10P, G10X, Mu05, Mu09, Mu10, Mu11, Mu15, Mu23, Mu26, Mu50, Mu51, Mu59, Mu61, CXX20	SRY	354 (non-invasive), 524 (mortality)
Ten different countries	**Alps – Dinara – Pindos Mountains and Carpathian Mountains**	**Opportunistic & systematic sample collection**	**Tissue & blood & bone & scat & hair**		**27 different loci screened**	**SRY, Amelogenin**	**1878 bears identified**

Based on the information presented from the various countries, workshop attendants participated in a fruitful discussion regarding the future of genetic research and conservation of brown bears in the region. Following, are the main points of interest discussed and/or decided upon during the workshop:

"*What are the general goals of genetic research on brown bears in the region?*"

The participants of the workshop agreed that the general goal of genetic research on brown bears in the region should be managing and protecting the species in the area on a population level. In order to achieve this general goal, three distinct levels of action were identified. These levels of action should follow and build upon each other.

Level 1

Establishment of a network of interested organizations in the genetic research of brown bears in Southeastern European countries (i.e. these include the bear populations of the Alps – Dinara – Pindos Mountains, the Carpathian Mountains and the Rila - Rodopi Mountains). This network should consist of at least one organization from the thirteen different countries and aim in establishing a constant flow of information on ongoing genetic projects while synchronizing also their research methodologies. Actions in Level 1 are consistent with the actions identified in the Guidelines for Population Level Management Plans for Large Carnivores (Linnell, Salvatori & Boitani, 2007).

Actions within this level are already underway: a network of contacts in most countries in the region has been established. Remaining countries will be contacted within the framework of this workshop (D. Huber will contact Bosnia & Herzegovina and Bulgaria; M. Paunović will contact Bosnia & Herzegovina and Montenegro, A.A. Karamanlidis will contact Albania) and their methodologies standardized. In order to achieve latter, a document describing the common guidelines for the genetic study of brown bears (*Ursus arctos*) of the Alps – Dinara - Pindos and Carpathian populations will be prepared.

Building upon the deliverables of this workshop, it is the intention of the network to prepare a presentation to the upcoming International Bear Association conference in Georgia in 2010 (tentative title: "Defining a transboundary strategy for the genetic research of brown bears (*Ursus arctos*) in the Alps – Dinara - Pindos –

and Carpathians Mountains", which could eventually develop into a review paper to the scientific journal "Ursus".

Level 2

Pilot studies should be carried out in all countries of the network, using the commonly identified methodology in Level 1. In this level of actions each country should aim in collecting a certain number of genetic samples; in countries with small populations this number should equal 10-15% of the minimum population estimate, while in countries with larger populations this number should be at least 30, unrelated individual bears. The aim of the genetic research at this level should be primarily to evaluate genetic diversity and gene flow, population structure and connectivity and to estimate effective population size. Such information should lead to the identification of Evolutionary Significant Units (ESUs) and Management Units (MUs).

Level 3

Creation of a permanent genetic monitoring system of brown bears in each country, throughout the entire network. Activities should be coordinated within each Evolutionary Significant Unit or Management Unit (depending on what has been identified in Level 2) and carried out through the support of national, transboundary or multinational projects.

Furthermore, an additional priority task was identified during the workshop. All participants agreed that the creation of a joint genetic database for individual brown bears is of utmost importance for the effective study of the species in the region. It was also decided that this task should be pursued outside the three aforementioned levels – for instance, through a direct application to the European Commission. Stefano Filacorda volunteered to coordinate the application while the rest of the group will provide the required input.

Implementing actions defined in Level 3 would require extensive logistic and financial support and could be achieved either through a single large project or various smaller national or transboundary projects. A single large project requires however extensive preparation and coordination between all members of the network. It was decided to define, as a minimum preparation for such an initiative,

the general framework of such a project/research initiative. This framework[1] is the following:

Tentative title: "Innovative use of genetic research in transboundary wildlife conservation and management"

Keywords: transboundary, genetics, conservation, management, important wildlife species, Evolutionary Significant Units (ESU), science, innovative research towards conservation.

1. Description of the general situation of wildlife genetic research in Europe (why this is needed) – habitat increasingly fragmented (infrastructure etc.), populations isolated, impossible to identify ESU without genetics, impossible to manage without understanding ESU or MU. Natural populations extend across national boundaries – collaboration is required and is in some cases already existing or starting.

2. What should be done (aims of the theme):

Development of new technology for genetic research, harmonize existing methodology between laboratories, application of genetics for conservation and management, create information-sharing systems for transboundary collaboration, integration of genetic research with »traditional« research methods. Genetics in medical research. Ecology.

"Genetics? What for?"

It was commonly accepted amongst workshop participants that the general goal of genetic research on brown bears in the region should be managing and protecting the species in the area on a population level. Taking for granted that molecular techniques are an ideal tool for doing this, what kind of information is it that we are seeking for? What kind of answers do we concretely expect to answer using this methodology? Following are the main topics of interest of genetic research identified for our study area; in brackets one can find applications of this kind of genetic research from other parts of the world:

[1] This framework was defined bearing in mind the guidelines for an application to the European Science Foundation; it is therefore broader than one would expect, if attempting to study only bears!

1. Genetic (population) indices, genetic diversity (Paetkau & Strobeck, 1994; Paetkau *et al.*, 1998b; Laikre, 1999; Waits *et al.*, 2000).
2. Geneflow, landscape genetics, population isolation and connectivity (Taberlet & Bouvet, 1994; Paetkau *et al.*, 1995; Taberlet *et al.*, 1995; Paetkau, Shields & Strobeck, 1998a; Waits *et al.*, 1998a; Waits *et al.*, 1998b; Manel *et al.*, 2003; Apps *et al.*, 2004; Manel *et al.*, 2004; Crompton *et al.*, 2008; Perez *et al.*, 2009).
3. Phylogenetics, phylogeography (Taberlet *et al.*, 1998; Waits *et al.*, 1998b; Sommer & Benecke, 2005; Valdiosera *et al.*, 2007; Valdiosera *et al.*, 2008).
4. Effective population size (Miller & Waits, 2003; Tallmon *et al.*, 2004).
5. Population size, trend, sex ratio, age, reproductive success (Taberlet *et al.*, 1997; Lorenzini *et al.*, 2004; Mowat *et al.*, 2005; Soldberg *et al.*, 2006; Zedrosser *et al.*, 2007; Kruckenhauser *et al.*, 2008; Kendall *et al.*, 2009).

2. "COMMON GUIDELINES FOR THE GENETIC STUDY OF BROWN BEARS (*Ursus arctos*) IN SOUTHEASTERN EUROPE"

Studying bears on a genetic level has become an integral and indispensable part of the research on the species. Testimony to this are the numerous publications that have appeared over the years; especially studies that combine genetic analysis with non-invasive sampling methods are becoming increasingly popular. The aim of the common research guidelines defined during the workshop is not to review all possible methodologies nor describe them in full detail, as most of this information has already been published and is readily accessible. The aim of this document is to provide a synopsis of the genetic studies that have been carried out in southeastern European countries and the methodologies they have developed and applied, with a special emphasis on innovative and successful research solutions. This document provides the minimum of information required in order to initiate independently and successfully a genetic study in the region and lists additional information sources. Such sources are provided either in form of published documents (i.e. as references in the reference list or as attached pdf documents) or as contact details of specific scientific expertise. The guidelines should ultimately help researchers involved in the genetic research of the species in the region adjust or alter their study design and/or methodologies with ones that proved especially successful in the area and to better understand their findings by comparing them with results from other research groups. For researchers that are currently not involved but are considering initiating a genetic study on brown bears the guidelines should provide research options to choose from that will lead to the application of a standardized methodology and make their study compatible to other research initiatives in the region.

2.1. Setting up a laboratory dedicated to noninvasive genetic samples

Before initiating any non-invasive genetic study a laboratory dedicated to this cause has to be set up or an agreement with an experienced lab made that will take over this part of this study. In the first case, and in order to guarantee the validity of results, several recommendations should be followed and conditions and requirements met. For laboratories dedicated to the analysis of non-invasive samples a physical separation between this room and the lab analyzing tissue samples is recommended. Furthermore, a separate room should be dedicated to PCR analysis and one for sequencing. Strict regimes regarding movement of personnel, equipment and material between laboratories in order to prevent contamination should be enforced.

All flow of material during analysis should be one-way, meaning that once any material leaves the room where material with low DNA concentrations is being handled, it should not return (e.g. PCR products should never return into the tissue lab, or anything from the tissue lab should never be brought into the non-invasive lab). In a non-invasive genetic lab, movement of personnel should be limited, with a rule that anyone who has been in any of the rooms where higher concentrations of DNA are being handled (tissue lab, PCR room, sequencer room) should not be allowed to enter the noninvasive laboratory until they have taken a shower and changed their clothes. All working surfaces in genetic laboratories should be regularly (usually daily) decontaminated with 10% bleach.

2.2. Organizing non-invasive genetic sample collection with volunteers

Monitoring shy and elusive animals, such as bears and getting meaningful results from this effort, usually requires a large number of non-invasive samples, which in turn may require a lot of manpower. While it is possible to carry out intensive monitoring of wildlife with professional staff, in many real-world situations this will not be feasible due to logistic and financial constraints. In many cases the help of motivated volunteers will be the preferred solution – their participation in any project will require however meticulous planning and preparation. Samples that have been collected in a wrong fashion might turn out to be useless, regardless of how good the lab or the researcher sitting behind the desk is. When preparing a project one should consider that the costs and time of organizing and implementing the sample collection might equal or exceed the costs of genotyping and data analysis. Therefore, considering the following points when deploying volunteers in the field should help save time, energy and money.

2.2.1. Information and motivation

While volunteers can be recruited through a number of very different channels (hunters, foresters, students, mountaineers etc.) there are always two critical points. First of all, volunteers have to know that a specific research project exists, and they have to find something in it that will motivate them to participate. In large-scale sampling efforts this will usually imply that a wide-ranging information campaign has preceded the actual sample collection. The size of the information campaign will depend directly on the size of the study area, but for any large-scale sampling effort one should plan at least 4 - 6 months of preparatory work. During this phase it is recommended to get as much personal contact to the volunteers as possible. Organizing lectures explaining the aims of the research and getting to communicate with a volunteer will be rewarded many times over once samples start coming in.

2.2.2. **Make participation simple!**

Volunteer participation in any project should be made as simple as possible and result for them in a rewarding and memorable experience! Here are some points to consider in order to achieve this:

- Sampling material (i.e. sample tubes, envelopes, instruction brochure, pencils to record sample data, data sheets etc.) should always be prepared by the project coordinator and made readily available (i.e. sampling material is always sent to volunteers, don't make them come and pick it up!).
- Project information and sampling material should look as professional as possible. A professional appearance will motivate volunteers to take their work seriously. One should therefore even consider hiring a professional designer to design the project material!
- Sampling guidelines should be simple and explained thoroughly during the preparatory phase of the project to all parties involved. Preferably, each volunteer should receive also a written copy of the project methodology and sampling guidelines (Fig. 1).

Figure 1: Cover of a brochure distributed to volunteers participating in 2007 in a large-scale sampling project of brown bears in Slovenia (© T. Skrbinšek).

- Make volunteers always feel "part of the team". Consider therefore providing some extra motivational "goodies" (e.g. T-shirts, caps, stickers etc.). Such "goodies" will help also recruit new volunteers.
- At the end of every sampling session the project coordinator (NOT the volunteers!) is responsible for collecting the samples.

2.2.3. **Stay in control during the sample collection**

During a prolonged sampling session one must be constantly in contact with the volunteers in order to demonstrate ones constant interest and remind them of the importance of their work. This should be done directly (calling and visiting is essential!) or indirectly, through constant media coverage or a project website.

2.2.4. **Provide feedback!**

This final step is undoubtedly one of the most important. Apart from the moral obligation of a research team towards the people who collected the raw material of their research, providing direct and indirect feedback will be essential in recruiting volunteers in the future. Within this context, scientific publications are not to be considered appropriate feedback as they are usually difficult to access and difficult to understand for volunteers (and scientists…). Indirect feedback could take the form of a web page, layman's and summary reports that are sent to volunteer groups and feature articles in magazines and newspapers. Direct feedback could take the form of lectures in local communities in the study area.

2.3. Data recording

Samples without the respective data about them are useless. Depending on research design and local circumstances the amount of data will vary. NOTICE: Recording a lot of data might not always be feasible and in certain cases (i.e. when volunteers are involved) also not desirable. However, the collection of a minimum amount of data should be guaranteed when starting any sampling procedure. In the case of non-invasive genetic sampling in the Alps – Dinara – Pindos and Carpathian Mountains, this should be:

- Date when the sample was found,
- who collected the sample,
- estimate of the sample's age,
- location at which the sample was found, preferably with GPS coordinates. As this might not always be possible in large-scale projects using volunteers, researchers should have made sure before starting the study that they have a way of determining where the sample was collected from.

This minimum amount of information should be recorded on a label that is stuck onto the sampling tube (when collecting scat) or envelope (when collecting hair). In this manner the data doesn't get separated from the sample, and the label guides the person collecting the sample to record all the necessary data. It is a good idea to use a dedicated thermal printer for labels and good paper labels. Such labels are much more durable and less prone to falling off when the sample is kept in a freezer, for a minimal additional cost. A printer for labels can also be used to print bar codes on waterproof and freezer-proof labels, providing permanent and reliable sample labeling (see also Section 2.6.1).

2.4. Collection of genetic samples

DNA can be extracted, with varying rates of success from a multitude of types of genetic samples. Genetic research in the Alps – Dinara - Pindos and Carpathian Mountains has focused so far on some of the most common types of samples, including hair, scat and tissue (Table 1).

Collection and storage of genetic samples is considered to be within the planning and setup of a scientific study one of the most, if not THE most important phase of the project! Mistakes carried out within this phase are most often irreversible and can lead to loss of valuable information. It goes therefore without saying that this phase has to be thoroughly planned and executed. Following are the practices that have been successfully deployed in the collection and storage of various types of genetic samples in the Alps – Dinara – Pindos and Carpathian Mountains study areas.

2.4.1. **Blood collection and storage**

In Slovenia and Greece, blood samples have been obtained from animals captured in telemetric studies. These samples are stored in Microtainer tubes with anticoagulant (EDTA) and are kept in a freezer at -20˚C.

2.4.2. **Hair collection and storage**

Hair can be collected in an opportunistic manner (i.e. from rub-trees, from bears killed in car accidents, from bears that cause damage to property, shed hair found on trails etc.) or most often in a systematic manner (i.e. using hair traps, or traps on rub-trees or power poles). Within latter approach one must distinguish hair sampling that uses bait from that that does not.

Hair traps using bait

Collection of hair using hair traps and bait was successfully carried out in the study area in Trentino (2003 - 2008). A study design outlined in previous DNA-based inventories in North America (Woods *et al.*, 1999; Boulanger *et al.*, 2002) was followed using a systematic grid. Considering the topography of the habitat, human presence, and home ranges of the translocated bears living in the area the grid cell size was small (4x4 km) and grid extent varied from 272 km^2 to 976 km^2. One hair trap was set up in each cell and baited using a mixture of ~50% rotten blood and fish scum. As a general guideline bait should be a lure and not food, in order to avoid behavioral response or habituation caused by a reward. Sites were visited for sample collection and lure replacement 14 days after initial setting, for 5-8 sampling sessions. Hair samples were collected using sterilized forceps and placed in coin envelopes

stored in zip lock bags with silica desiccant and stored at room temperature (Roon, Waits & Kendall, 2003).

Hair traps without bait

Hair sampling in the southwestern Balkans has followed a different methodological approach and has taken advantage of the marking and rubbing behavior of brown bears on poles of the electricity and telephone network (Fig. 2).

Figure 2: A brown bear in Greece in a "tender" encounter with a power pole. Brown bears in Greece, Albania and F.Y.R. Macedonia have been observed to frequently mark and rub on poles of the electricity and telephone network (© Krambokoukis/ARCTUROS)

This behavior has been used to develop a method for documenting the presence and carrying out non-invasive studies of brown bears in the region (Karamanlidis *et al.*, 2007). Since 2003 more than 5000 poles have been inspected in the study area and

classified according to the freshness and amount of bear evidence found on them (Fig. 3., Table 2).

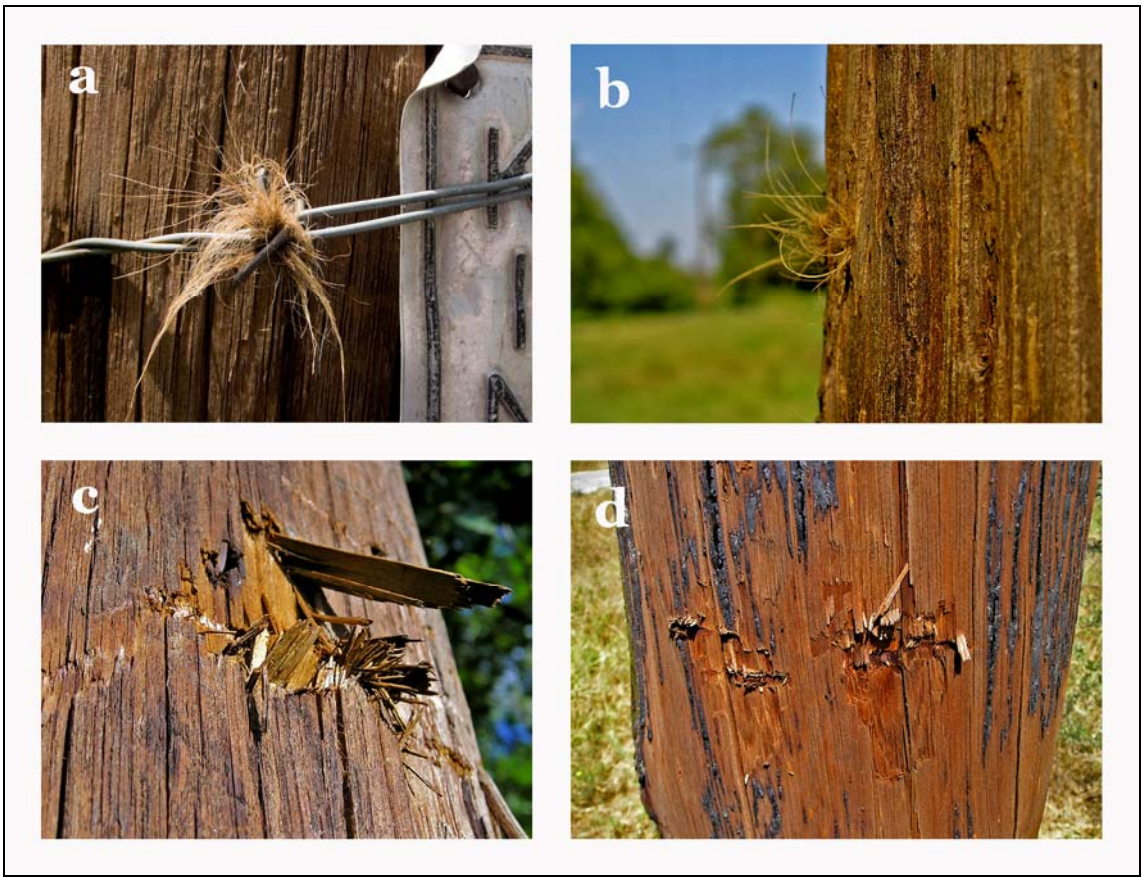

Figure 3: Deterioration rate of bear signs in the field (a: Stage 1 – Hair is long, curly and brownish, b: Stage 2 – Hair is short and blond, c: Stage 1: Big difference in colouration between newer and older marks and small pieces of wood sticking out of the pole, d: Small difference in colouration between newer and older marks on the pole (© Karamanlidis/ARCTUROS).

Table 2: Number of power poles used in six sampling networks for genetic sampling and their intensity of use by brown bears (use-category I: low rubbing activity; use-category II: low marking activity; use-category III: medium marking and rubbing activity; use-category IV: heavy marking activity; use-category V: heavy marking and rubbing activity (see Karamanlidis *et al.* 2007 for more information on these categories) in the southwestern Balkans (April – May 2008). *N*/100km² is a density index of the sampling network, calculated as the number of sampling power poles divided by the size of the study area in 100km².

Study area	**Category I**		**Category II**		**Category III**		**Category IV**		**Category V**		**Nr. poles/area**
	N	*N*/100km²	*N*	*N*/100km²	*N*	*N*/100km²	*N*	*N*/100km²	*N*	*N*/100km²	
Albania	0	0	2	0.4	0	0	2	0.4	2	0.4	**6**
FYROM	3	0.05	3	0.05	18	0.3	1	0.01	8	0.1	**33**
Greece/Florina	5	0.5	8	1.4	25	2.4	2	0.2	12	1.1	**52**
Greece/Grammos	1	0.1	3	0.4	20	2.7	2	0.2	46	6.2	**72**
Greece/Grevena	0	0	2	0.3	19	3.0	3	0.4	30	4.7	**54**
Greece/Trikala	3	0.4	10	1.4	28	3.9	3	0.4	11	1.5	**55**
Overall Nr. poles	**12**		**28**		**110**		**13**		**109**		**272**

Following the initial inspection, 272 of these poles were selected to create a large-scale sampling network and since the beginning of 2008 are inspected monthly. In order to minimize the chance of a bear rubbing against a pole without leaving hair behind and its visit going undetected, poles have been fitted with barbed wires. A single piece of barbed wire was fitted to each pole, reaching from the ground to a height of approximately 2.0 m. Wraps around the pole were distanced approximately 30 cm from each other.

2.4.3. **Scat collection and storage**

Despite the initial reluctance to use scats on a wide scale as genetic material, due to small amounts of extracted DNA and increased costs, recent methodological improvements have made scats an increasingly popular sample type. However, even so, collecting and storing scat samples is not as straightforward as procedures in hair sampling; following are some critical points that should be taken in account:

- Currently the most effective and simple method of storing scat samples seems to be in 95 - 96% ethanol at room temperature or refrigerated (4°C) (Frantzen *et al.*, 1998; Murphy *et al.*, 2002; Piggott & Taylor, 2003). For long-time storage, this can be augmented by storing samples in a freezer (-20°C).
- Sample tubes have to be inexpensive and yet durable enough so that the content is not spilled (this is especially important if sampling is done by volunteers). The recommendation of the Slovenian team is the cheap Greiner 50 ml centrifuge tube (No. 210261).
- The actual collection of a scat might be a little bit tricky! If too much scat is put into the sample tube, the amount of ethanol will not be sufficient to conserve the scat and DNA will continue to degrade. Ethanol has the highest bactericidal activity and best penetration of material in 70% concentration. Therefore teams in Slovenia pre-filled sample jars to 3/5 with ethanol, which made people collecting the samples reluctant to add too much as this would cause spillage. In Trentino, ~10mL of scat sample from the outside surface of the feces (Stenglein *et al.*, In press) was collected and preserved in 40mL 95% ethanol.

- Scats should be collected using a different "tool" each time in order to prevent cross-contamination. In environmentally aware projects, such tools are readily available for free in the forest (Fig. 4). The remaining scat must be removed or clearly marked after the sample is collected to prevent double collection.

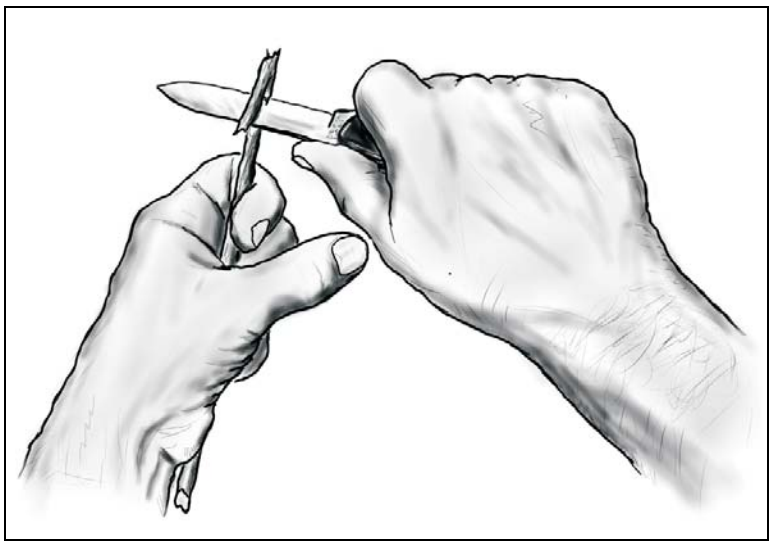

Figure 4: A good and simple tool for scat collection is a twig cut-off flat on one side. After the collection, this "tool" is thrown away (© T. Skrbinšek).

- Which part of a scat to collect has been recently a subject of increased scientific interest. Logic has it that the best part of the scat to take as a sample would be the most protected part with as many epithelial cells as possible. If there is mucous present, it should be taken as it contains a lot of epithelial cells. Drying should conserve the DNA, while washing (rain) and direct UV radiation should degrade it. By this logic the sample material should be taken from the surface (Fig. 5) (fast drying), but not where the scat is in contact with the ground (usually moist) and not from the top of the scat (more exposed to washing by rain). These assumptions have been recently verified in experimental research (Stenglein *et al.*, In press).

Figure 5: Collection of a bear scat for genetic analysis (© T. Skrbinšek).

2.4.4. **Tissue collection and storage**

Systematic tissue collection is very important, especially if bear mortality is readily detected, as it can, over the years, provide a "genetic history" of the population. In countries like Slovenia and Croatia tissue samples have been collected in a systematic manner, in cooperation with the Slovenian Forestry Service and Croatian hunting organizations respectively (general guidelines on sample collection from volunteers are provided in Section 2.2.2), within the restrictions of the annual hunting quotas. In Greece, tissue samples are collected opportunistically from dead animals (i.e. bear – vehicle collisions, poached individuals) or animals captured for scientific purposes. In Slovenia tissue samples (~4 cm^3 of muscle or skin) from every known mortality were stored in 50 ml screw-cap tubes prefilled to 3/5 with 96% ethanol. Similarly, in Croatia tissue samples were stored in 96% ethanol in 15 mL tubes, with a sample to ethanol volume ratio approximately 1:10 and kept in a refrigerator at either -20°C (preferably) or +4°C (when lacking freezer space). The sample tubes for tissue should be equipped with paper labels on which the information about the samples are recorded. Apart from the data commonly recorded (see also Section 2.3) the sex of the animal and its estimated age and weight should also be recorded.

2.4.5. **Bone collection and storage**

Bones should be stored dry in a zip lock bag with silica gel.

2.5. Sampling design

Several factors influence the number of genetic samples collected and the amount of DNA extracted and ultimately play a significant role in the success and viability of a genetic monitoring project on bears. Following are some of the most important amongst them.

2.5.1. Sampling period

"When should sampling occur?" Sampling success depends on sample type (e.g. hair vs. scat) as well as a number of local parameters (i.e. anthropogenic, environmental, behavior of the bear etc.); thus optimal sampling periods will differ between different study areas. It is therefore advisable to carry out, if possible before initiating a long-term non-invasive project, a pilot project in each study area respectively that will account for such parameters.

Optimal sampling period for hair sampling

In a non-invasive genetic sampling pilot study carried out in Trentino, the most successful time period for hair sampling was mid May - mid August. During this time, more samples of higher DNA quality were collected and more individuals were detected compared to sampling sessions during September - October (De Barba, 2009). Hair trapping in North America is also performed approximately in May - August (Mowat & Strobeck, 2000; Poole, Mowat & Fear, 2001). In a similar pilot project carried out in Northern Greece, the optimal period for hair sampling was between the end of April and mid June; collecting hair from power poles was directly associated to the marking behavior of brown bears, which in turn was influenced by the mating behavior of the species (Karamanlidis et al. unpublished data).

Optimal sampling period for scat sampling

There is some literature available that deals with the effects of the season of sample collection (Piggott, 2004) and sample age (Murphy *et al.*, 2006; Murphy *et al.*, 2007). In the Northern Dinarics, in Slovenia, scat samples collected in late summer and autumn had a much higher genotyping success rate than samples collected in spring and early summer. Also, success rate of samples containing beech nuts was higher than that of samples containing other food items (Skrbinšek *et al.*, unpublished data).

2.5.2. **Sampling frequency**

"How often should sampling occur?" Again, sampling frequency will depend on sample type and local parameters.

Optimal sampling frequency for hair sampling

Temporal frequency of hair sampling should affect DNA quality, as more time samples remain in the field the more they are affected by environmental agents that can degrade the DNA. I.e. systematic sampling for bear hair in Greece carried out using 30-day sampling sessions resulted in genotyping success rates of ~72 - 82% (Karamanlidis et al. unpublished data). This rate fell at 25% for samples collected when remaining >4 weeks in the field. Extensive field tests in Greece indicate that the deterioration rate of hair follows a well-defined pattern (Table 3) and that hair freshness can be easily and accurately evaluated by experienced field researchers.

In Trentino in comparison (approximately 1000km north of the study area in Greece), genotyping success was ~70 - 80% during sampling sessions of 14 days (De Barba 2009). In areas therefore with higher (summer) precipitations a shorter sampling session should be considered.

Table 3: Deterioration rate of hair samples in field conditions in Greece (Karamanlidis *et al.*, 2007)

Type of sign	Stage	Time since deposition	Characteristic features
Hair deposits	I	1-2 months	Long, curly, brownish hair; found in locks on the surface of the pole. Hair is flexible and breaks difficultly.
	II	3-6 months	Short, straight, bleached out hair; found as individual hairs on the surface of the pole. Hair is stiff and breaks easily.

Optimal sampling period and frequency for scat sampling

The same general principles and guidelines that apply for hair collection apply also for scats, i.e. the fresher the scat the better. In Slovenia, scat samples from the Northern Dinarics bear population that were judged to be less than 1 day old had over 90% genotyping success rates. This rate dropped rapidly, and was below 50% for samples subjectively judged to be 4-5 days old. The Slovenian team decided therefore not to collect samples from scats that appeared to be older than 5 days. The Croatian team has come to similar results during their research.

Estimating therefore scat freshness is an essential step in the sampling process that can cull unsuitable samples and prevent unnecessary loss of valuable energy, time and funds. In Slovenia, scat-collecting teams were provided with general instructions on how to estimate the freshness of a scat. Fresh scats have a content-specific smell and mucous is present. In dry and warm weather scats can dry rapidly, but they still retain some smell and have no "holes" from insects and their larvae. If there is a lot of green plant material, scats turn from green to black from the surface

towards the center in a couple of days. Insect larvae can be present after a couple of days, but they exit the scat again in a couple of days (in summer, as soon as after a week) leaving behind little "holes". Old scats usually smell like soil, often have "holes" if the larvae have already left, and have no visible mucous. Old scats are usually dry, but can be moist after rain although they will dry rapidly. In either case there is no mucous present.

2.5.3. **Sampling intensity**

The number of hair traps to set up or transects to walk will depend on the topography of the study area and the home ranges of the bears. Enough hair traps or transects should be established in order to maximize the probability that a bear will encounter a hair trap or a transect. For hair traps this is usually done by overlapping to the study area a systematic grid of the proper cell size (i.e. in Trentino a cell size of 4x4 km was used considering the small home ranges of female bears and the rugged topography). Where power poles are used for hair sampling a density of > 1.0 poles/100km^2 of Category V (i.e. the most heavily used poles) is recommended. A similar approach can also be applied to transect sampling.

2.5.4. **Sampling design for capture – mark – recapture modeling and abundance estimates**

While sampling design is always important, it is seldom as critical as in the case of capture-mark-recapture (CMR) studies, especially if the research goal is an abundance estimate. While CMR modeling is becoming extremely flexible through development of new models and software packages, all this becomes useless unless the data has not been collected in a manner that satisfies the modeling assumptions as much as possible. The text provided here summarizes the experiences obtained in two projects that employed non-invasive sampling of scats in Slovenia. One was a pilot project, where scats were collected over two small areas (170 and 230 km^2), and the other a large-scale effort to estimate the total number of brown bears in Slovenia with over 1000 volunteers participating in a very intensive sampling effort over the entire bear range (approximately 6000 km^2).

- *Understanding the assumptions of CMR models*

This point can't be overstressed. Study designs that violate CMR assumptions and samples that were collected in a false manner will most likely result in low-quality data. A good resource for mark-recapture analysis is the "Handbook of Capture-Recapture Analysis" by S.C. Amstrup *et al.* (Princeton University Press, 2005). Another very good, and freely available book is "Program MARK: A Gentle Introduction" by E. Cooch and G. White. The book is regularly updated, spans more than 800 pages and is freely available at *http://www.phidot.org/software/mark/docs/book/*. It provides a short but concise overview of the theoretical background and hands-on examples using Program MARK, which is probably the most comprehensive CMR analysis software currently available (White & Burnham, 1999). It is highly advisable to work through (and understand!) the chapters 1-7 before contemplating any sample collection. There are also several recent studies where non-invasive genetic sampling has been used to estimate abundance of brown bears (Soldberg *et al.*, 2006; Kendall *et al.*, 2008), providing sufficient background for future research.

- *Number of samples required for CMR studies*

The number of samples required for a CMR study will depend on the goal of the study. If the goal of the study is an abundance estimate then the rule of thumb is to aim at collecting 2.5 — 3 times the number of samples of the "assumed" number of animals present in the researched population (Soldberg *et al.*, 2006). A better understanding of the required sampling effort can be achieved with a power analysis using MARK simulation models (White & Burnham 1999). Several sampling scenarios can be simulated, and the results analyzed to understand what confidence intervals to expect from a certain number of successfully genotyped samples. A point to consider is the expected genotyping success rate, which should be used to correct the estimated number of required samples. In Slovenia, genotyping success rate from scats, when only fresh samples were collected and the sampling was done in autumn, was 88%. If only reasonably fresh samples are collected, the expected success rate should be at least around 70%, although a more conservative estimate of 60-65% should be used for planning, if no experience of non-invasive genotyping from the planned study area exists. A recent review of amplification success in different species is provided in Broquet *et al.* (2007).

- *Modern CMR design*

The possibilities of CMR modeling go far beyond abundance estimates. If done systematically over several years, it is possible to get an understanding of recruitment and survival. If there are several areas with limited migration possibilities in between, one could estimate migration rates. Ultimately, this can prove to be much more valuable for conservation than just the abundance estimate. Detailed information on these issues is provided in the "robust design", "multi strata" and Pradel models in the Mark book (Cooch & White, 2009).

2.6. Labeling and tracking of samples

When samples reach the lab, it is important to label and store them in a reliable manner, and to track them as they go through the analysis, so that sample mix-ups do not occur. Here are some points to consider when labeling and tracking samples.

2.6.1. Labels and labeling

Samples without labels are absolutely useless; a reliable, indelible, permanent labeling of samples is therefore imperative. Labeling with a permanent marker does work, but if any alcohol from the sample tube is spilled on the label, it will get erased. It is therefore recommended to use a thermal printer for printing labels. This provides several advantages:

- Printing on a wide variety of materials, including waterproof or freezer proof plastic labels is possible. Such labels are very stable and will not fall off.
- Labels are printed in a long ribbon, and tools for sticking them on tubes can be purchased or constructed, making labeling much easier and faster.
- Even for paper labels that can be written on using a pencil, it is possible to get tougher labels with better glue for thermal printers. Also, the print done by a thermal printer is much more stable than when a regular laser printer is used. Ink jet is not an option.

2.6.2. Barcodes and bar-coding

Barcodes offer a simple method for labeling your samples, and prevent typing errors. Any number or text can be transformed into a barcode that can be later read by a barcode scanner. It is as simple as finding a barcode font on the internet, installing it and changing the font properties of the label text into the barcode font. In Slovenia barcodes are printed on small plastic, waterproof and freeze proof labels together with a human-readable code. Two labels are stuck on each sample tube, one on the cap and one on the tube, just in case one gets loose.

A current limitation of the barcodes is that they need to be of reasonable size (at least 0.5 × 1 cm) for a barcode scanner to read them, and the surface needs to be reasonably flat. This becomes a problem if extracted DNA is aliquoted into 0.2 ml

Eppendorf tubes to be used with a multichannel pipette, as these tubes are too small. This may in the future be solved through use of RfID chips, which are also becoming financially accessible.

2.6.3. **Sample codes**

Coding of samples is an important issue. As tempting as it is to have as many data as possible already in the sample code, somewhere down the line it might be necessary to hand-write this code. If laboratory procedures dictate to aliquot the extracted DNA into 0.2 ml tubes (which can't have barcodes as they are too small) that can be arranged into a 96-sample rack and pipetted using a multichannel pipette, one really can't write more than 4 characters, and so this should be the limit of the sample code. If the codes are hand-written ambiguous characters should be excluded. I.e., letter O and digit zero, letter S and digit 5, B and 8 etc. can get easily mixed up when hand written and should be avoided. In Slovenia a 3-character code capable of encoding 10,648 samples, using the unambiguous characters "012345678ACEFHJKLMPTUX" is being used. A simple code for use in MS Excel for transforming integers into the 3-character code is presented in the Appendix A.

2.6.4. **Minimizing manual data entry**

Manual data entry should be kept to a minimum in order to avoid typing errors. It is recommended to print out a large number of waterproof / freeze proof labels with unique codes and stick them on all sample tubes or envelopes either before the material is distributed to the field crew, or immediately when the samples arrive to the lab. When the data is recorded or the sample manipulated, a barcode is scanned, avoiding the dangers of manual data entry.

2.6.5. **Photo documentation**

It is recommended to photograph sample arrangements in each critical step of the laboratory analysis. These photographs should be later on routinely re-checked to see if they conform to the planned sample arrangement, in order to detect potential sample mixups.

2.7. DNA extraction

Methods for DNA extraction differ depending on the type of sample. Following are the methods used for extracting DNA in the various projects and types of samples.

2.7.1. Blood

DNA extraction from blood samples is possible using the GeneElute™ Mammalian Genomic DNA Miniprep Kit (Sigma) according to the instructions of the extraction kit manufacturer.

2.7.2. Hair

DNA extractions from hair samples are performed in Greece and Trentino using the DNeasy Blood & Tissue kits (QIAGEN, Hilden, Germany) following the manufacturer' s instructions. All extractions take place in a building in which amplified DNA has never been handled. In Slovenia, DNA extraction is done using the GeneElute™ Mammalian Genomic DNA Miniprep Kit (Sigma) according to the manufacturer's instructions. Hair samples are left in Lysis T buffer and proteinase K over night at 56˚C. Despite using different kits, all groups aim at using ten guard hairs where available. In Greece, bear DNA content is checked by PCR with a single primer pair (G10J) – negative samples are discarded and positive samples genotyped.

2.7.3. Scat

Fecal samples in Croatia, Greece, Slovenia and Trentino are extracted using the Qiagen QIAmp™ DNA Stool Mini Kit for DNA extraction, according to the manufacturer's protocol. 0.1 – 0.2 ml of feces is used in a room dedicated to processing low quantity DNA samples. In Slovenia a part of each fecal sample is taken out of the storage tube, spread over the surface of a disposable Petri dish and left for a few minutes for the ethanol to evaporate. Large particles (large parts of leaves, hair, corn seeds etc.) are separated, and the remaining fine material with a large surface to volume ratio used for the extraction. It is recommended to use dedicated chemicals and pipettors for DNA extractions. Each set of extractions should include a negative control in order to check for contamination. In Croatia DNA content in extracts is

being checked by PCR with a single primer pair (Mu51) and agarose gel electrophoresis. Negative samples are discarded and positive samples genotyped.

2.7.4. **Tissue**

In Slovenia tissue samples are stored in 96% ethanol in a freezer at -20˚C. Isolation of DNA is done using the GeneElute™ Mammalian Genomic DNA Miniprep Kit (Sigma) according to the manufacturer's instructions. In Croatia DNA from muscle tissue is extracted using the Wizard Genomic DNA Purification Kit (Promega, USA) and following the manufacturer's protocol. Each set of extractions includes a negative control in order to check for contamination.

2.7.5. **Bone**

Successful extraction of DNA from bones can be performed by grinding the material in a swinging ball mill (Retsch MM400) und subsequent DNA extraction with the Gen-IAL First DNA extraction kit following the manufacturers' protocol for DNA preparation from bones and teeth adapted for small volumes.

2.8. Microsatellite analysis

Microsatellite analysis will depend on various parameters, such as research questions, lab expertise and available equipment and is the reason why laboratory protocols differ so much amongst the various groups currently involved in the genetic research of brown bears in the Alps – Dinara – Pindos and Carpathian Mountains. Following, three successful examples are presented.

2.8.1. Croatia

- **Tissue samples** were genotyped by amplifying 13 microsatellite loci [Mu10, Mu23, Mu50, Mu51, Mu59 (Taberlet *et al.*, 1997), G10B, G1D, G10L (Paetkau & Strobeck, 1994), G10C, G10M, G10P, G10X (Paetkau *et al.*, 1995), G10J (Paetkau *et al.*, 1998b) and the sex-specific SRY locus by PCR and using fluorescently end-labeled primers. The loci were amplified in five multiplex PCR amplifications: (1) G1D, Mu10, Mu50; (2) Mu23, Mu59; (3) G10L, Mu51, SRY; (4) G10B, G10C, G10M; (5) G10J, G10P, G10X. Each PCR consisted of a 10 µl volume of 1X Qiagen Master Mix, 0.5X Q solution (both Qiagen Multiplex PCR Kit, Qiagen, USA), 0.2 µM of forward and reverse primer, RNase free water (Qiagen, USA) and 1 µl template DNA. Amplifications were performed in a GeneAmp PCR System 2700 (Applied Biosystems) under the following conditions: 94 °C for 15 min., 30 cycles of 30 s denaturing at 94 °C, 90 s annealing at 60 °C, 1 min. extension at 72 °C, and 30 min. at 60 °C as a final extension step. Following amplification, 1 µl of PCR products for each sample were pooled in two mixtures, the first one containing products of PCRs 1, 2 and 3, the second of PCRs 4 and 5. The PCR products were combined so that all loci could be scored in two runs. One µl of the prepared mixture, either the first or the second one, was added to a 11 µl mix of 10.5 µl deionised formamide (Hi-Di Formamide, Applied Biosystems) and 0.5 µl ROX 350 (Applied Biosystems), and loaded on a four-capillary genetic analyser ABI3100-Avant (Applied Biosystems). The runs were analyzed and loci scored using Genemapper Software package v.3.5 (Applied Biosystems).

- **Scat samples** were genotyped by amplifying 6 microsatellite loci and the SRY locus in two multiplex PCR reactions: (1) Mu23, Mu51, Mu59, G10L; (2) Mu10,

Mu50, SRY. Reaction volume was 10 µL, containing 1X Qiagen Master Mix, 0.5X Q solution (both Qiagen Multiplex PCR Kit, Qiagen, USA), 0.2 µM of forward and reverse primer, RNase free water (Qiagen, USA) and 2 µl template DNA. Amplifications were performed in a GeneAmp PCR System 2700 (Applied Biosystems) and the temperature profile was 15 min at 94°C; followed by 45 cycles: 30 s at 94°C, 90 s at 60°C and 60 s at 72°C; final extension 10 min at 60°C. For each sample, the PCR products were pooled together so that all loci could be scored in one run. The products were resolved by capillary electrophoresis in a ABI3100-Avant genetic analyser as described for tissue samples. The runs were analyzed and loci scored using Genemapper Software package v.3.5 (Applied Biosystems). A multitube approach was used and up to eight (and in some cases up to twelve) PCR repetitions were carried out to obtain reliable genotypes; these were later on checked with RELIOTYPE software (Miller, Joyce & Waits, 2002).

2.8.2. **Greece**

In order to test the polymorphism of genetic loci in the southwestern Balkans 49 hair samples have been screened at 21 markers (Ostrander, Sprague & Rine, 1993; Paetkau *et al.*, 1995; Taberlet *et al.*, 1997; Paetkau *et al.*, 1998a; Kitahara *et al.*, 2000; Breen *et al.*, 2001). Thermal cycling was performed using a MJ Research PTC100 thermocycler with 96 well 'Gold' blocks. PCR buffers and conditions were according to (Paetkau *et al.*, 1998a), except that markers were not co-amplified as co-amplification reduced success rates for hair samples. 3µl of a total extract volume of 125µl per PCR reaction were used, except during error-checking when 5µl was used. [MgCl2] was 2.0 mM for all markers except MU26 (1.5mM), MSUT-2 (1.5mM) and G10J (1.8mM). Microsatellite analysis used ABI's four color detection system; an automated sequencer (ABI 310) was used and genotypes were determined using ABI Genescan and Genotyper software. Error-checking and general quality assurance followed strictly the guidelines of Paetkau (2003).

2.8.3. **Slovenia**

The analysis protocol for scats is explained in detail in Skrbinšek et al. (in press). All 14 loci (Table 5, Annex C) in Slovenia are multiplexed in a single PCR reaction. For all PCRs Qiagen Multiplex PCR kits are used. Ten µl reactions are prepared – 5 µl of Qiagen Mastermix, 1 µl of Q solution, 2 µl of template DNA, and 2 µl of water and primers to obtain the appropriate concentration in the final solution. All primers are premixed in a primer mastermix for easier pippeting. The cycling regime is a 15-minute initial denaturation at 95 °C, followed by 38 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 90 seconds and elongation at 72 °C for 60 seconds. PCR is finished with a 30-minutes final elongation step at 60 °C.

Tissue samples are amplified at 22 microsatellite loci and one sex determination locus (Table 6, Annex C) in three multiplexes (A, C and D) with two different cycling regimes. Ten µl reactions are prepared – 5 µl of Qiagen Mastermix, 1 µl of Q solution, 1 µl of template DNA, and 3 µl of UHQ water and primers mixture to obtain the appropriate concentration in the final solution. The cycling regime for multiplexes A and C is a 15-minute initial denaturation at 95 °C, followed by 29 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 90 seconds and elongation at 72 °C for 60 seconds. PCR is finished with a 30 minutes final elongation step at 60 °C. The cycling regime for multiplex D differs only in the annealing temperature, which is 49.5°C. The same PCR protocol is used for hair samples except for the number of cycles, which is increased to 35.

A mixture of 1 µl of the PCR product, 0.25 µl of GS500LIZ size standard (Applied Biosystems) and 8.75 µl of formamide is loaded on an automated sequencer for fragment analysis.

A dedicated laboratory for DNA extraction and PCR has been setup, strict rules regarding movement of personnel, equipment and material between laboratories to prevent contamination are enforced, and rigorous cleaning and decontamination regimes are applied. Pipette tips with aerosol barriers are used for all liquid transfers. A negative control extraction is done with each batch of 11 - 23 samples, and later analyzed downstream with the samples. Three negative controls are used on each 96 well PCR plate to detect possible contamination. Manual entry of data is kept to a minimum in order to avoid typing errors. Bar codes are used to track samples, and photo documented and later rechecked in order to prevent sample mix-up.

2.9. Sex determination

It is possible to identify the sex of individual bears either through the analysis of the amelogenin gene (Ennis & Gallagher, 1994) or the analysis of the SRY gene (Bellemain & Taberlet, 2004), which has the advantage of being carnivore-specific and less prone to miss-assignments if the bear ate meat of a male herbivore. In Croatia the sex specific marker SRY was amplified, depending on sample type, together with two microsatellite loci.

2.10. Ensuring genotype reliability and error checking

An important step in the analysis of genetic samples is ensuring genotype reliability and error checking. The following example from Slovenia shows how this can be done:

Ten percent of tissue samples were randomly selected (Pompanon *et al.* 2005) and the genotyping processes repeated to determine error rates. DNA extractions were not repeated. With fecal samples a multitube-based (Taberlet *et al.* 1996) genotyping procedure similar to the one proposed by Frantz *et al.* (2003) and modified by Adams and Waits (2007) was used to decide when to accept a genotype or discard a sample. The procedure was modified to accept a genotype if it was matching a genotype of an already reliably genotyped reference sample, with a constraint that the maximum likelihood estimated reliability (Miller, Joyce, & Waits 2002) of the reference sample must have been at least 0.95. For samples that didn't match any other sample, this threshold was set at 0.99. It was possible to determine the expected numbers of mismatching loci between different animals by genotyping a large number of tissue samples of known individuals. If two samples mismatched at a lower number of loci than expected between different animals, they were considered as belonging to the same animal and the match was accepted (2 allelic dropout mismatches in the large-scale study where 12 microsatellite loci were used for genotyping). Mismatches that would be caused by allelic dropout were treated separately from the mismatches that could only be caused by false alleles, as the latter are significantly less common.

The methods recommended by Broquet & Petit (2004) were used to estimate the frequency of allelic dropouts and false alleles, and a quality index was calculated

for each sample following the method described by Miquel *et al.* (2006). Samples with a quality index below 0.4 that did not match any other sample were discarded.

2.11. Data analysis

Various programs have been used by the different research groups in order to answer different research questions. Following, a summary of this software is presented:

- Estimating genotype reliability and the number of replicates needed to reach 99% accuracy can be achieved using RELIOTYPE (Miller *et al.*, 2002).
- Matching sample genotypes to references can be achieved using GENALEX (Peakall & Smouse, 2006).
- Testing for evidence of recent bottlenecks events from allele frequency data can be achieved using BOTTLENECK v 1.2.02 (Piry, Luikart & Cornuet, 1997).
- Estimating heterozygocity, number of alleles per locus, $P_{ID(sib)}$ and performing parentage assignment can be achieved using GIMLET (Valiere, 2002).
- Examining mismatch probability distributions can be achieved using MM-Dist (Kalinowski, Sawaya & Taper, 2006).
- Testing for Hardy-Weinberg Equilibrium and LE can be achieved using GENEPOP (Raymond & Rousset, 1995).
- Estimating Fis and allelic richness can be achieved using FSTAT (Goudet, 1995).
- Estimating population parameters using capture-mark-recapture approaches can be achieved with program MARK (White & Burnham, 1999).
- Single-session population estimates from non-invasive genetic sampling data can be obtained with CAPWIRE (Miller, Joyce & Waits, 2005).

2.12. From the field to the lab to the computer – an example of sample tracking, labeling and handling from a large-scale genetic study in Slovenia

Each sample tube was labeled with unique 3-character identifiers on two waterproof and freeze proof plastic labels (one on the cap and one on the tube), and prefilled with ethanol before it was handed out in the field. Another 10 × 10 cm paper label with a form to record the data about the sample was also stuck on the sample tube (Fig. 6), so that all the data about the sample remained with the sample. The form on the label was kept as simple as possible.

Figure 6: Sample label / form used in a non-invasive genetic study of brown bears in Slovenia (© T. Skrbinšek)

A sampling package was prepared for each volunteer collecting samples in the field – a plastic bag with 3 sample tubes, an information brochure explaining the background of the project and the project methodology, and a graphite pencil for recording data (graphite pencils don' t get erased if alcohol is spilled over the label). A batch of these packages was prepared for each participating organization (105 hunting clubs, 4 special purpose hunting reserves and 6 regional Forest Service offices), and barcodes of all sample tubes were scanned to have an exact record of where each sample tube went.

When a sample was returned to the lab, its barcode was scanned and all the data written on the label entered into a Microsoft Access database. When the sample

was to be extracted, it was scanned again and the extraction data entered into the same database. 100 µl of the extracted DNA was aliquoted in a 0.2 ml Eppendorf tube and used in the downstream analysis, while the remaining 100 µl aliquot was stored as a backup. Since 0.2 ml Eppendorf tubes are too small to use barcodes, they were hand-labeled in two places, on the cap and on the body, and a photograph of arranged samples and arranged 0.2 ml tubes was taken for future detection of possible mislabeling.

To minimize the possibility of a sample mixup during PCR setup, a plan of the sample layout was printed directly from the database for each 96-well PCR plate. Aliquots of template DNA in 0.2 ml Eppendorf tubes were arranged in a 96-hole stand according to the layout, and the DNA transferred using a multichannel pipette. The actual arrangement of the sample aliquots in the stand was then photographed, and the photograph later rechecked against the printed layout to ensure the correct arrangement of samples. An analysis protocol for the automatic sequencer was automatically prepared from the sample layout, so that the sample codes and the exact arrangement of samples on the PCR plate were directly imported into the sequencer's analysis software without any manual data entry.

When the final fragment analysis results were produced in the GeneMapper, they were directly imported into the relational database, providing automatic tracking of the entire collection and analysis history of each sample. A number of software tools were programmed directly into the database. The database automatically created consensus genotypes and analysis statistics for each locus and allele, calculated error estimates (Broquet & Petit, 2004), basic genetic diversity indices (Ho, He, A), probabilities of identity (Waits, Luikart & Taberlet, 2001), quality indices (Miquel *et al.*, 2006), and summarized the analysis history of each sample. It also searched for matching samples, provided export and import for Reliotype (Miller *et al.*, 2002), provided connectivity with GIS software, export into GENEPOP format, and prepared import files for mark-recapture analysis in Program MARK (White & Burnham, 1999). In this manner we avoided most of the manual data manipulation usually required to use various programs needed for analysis. Each of these programs typically requires a very specifically formatted input file, creating ample opportunities for errors when the data is manually rearranged using spreadsheet software.

3. CONCLUSIONS

Following research priorities for future genetic research on brown bears in the Alps – Dinara – Pindos and Carpathian Mountains have been identified:

1. Each country finds the most economical manner to provide reliable analysis of the samples, either using local facilities, facilities of project partners or a commercial laboratory.
2. Each country should develop capacities for data analysis and interpretation. Partners with expert knowledge in specific topics will provide the guidelines and/or expertise. Workshops dealing with specific issues will be organized. We will provide data exchange and develop analysis strategies to get population-level results.
3. Each country elaborates a plan for sample collection.
4. Each country tries to collect a sample from every dead animal.
5. Each country samples all the animals in captivity.

4. LITERATURE CITED

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5. ANNEX A

Table 4: Contact details of the individuals and organizations participating in the network for the genetic study of the brown bear in the Alps – Dinara – Pindos and Carpathian Mountains

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6. ANNEX B

Microsoft excel program for transforming integer sample numbers into a 3-character code for sample labelling

This program will encode an integer from 0 to 10647 into a 3-character code for labelling samples. Characters used for the code are inserted as an argument when called from Excel:

calculatecode(Number;"code string").

Example:

To transform the number 4350 into the three character code using the unambiguous characters "012345678ACEFHJKLMPTUX", use the following command within a spreadsheet cell:

```
=calculatecode(4350;"012345678ACEFHJKLMPTUX")
```

The result for this number is "4XL". In this manner it is easy to transform a large number of integers into an unambiguous 3 character code. Before use, the following code needs to be copy/pasted into a Visual Basic project within the same Microsoft Excel spreadsheet:

```
Public Function CalculateCode(iNumber As Integer, sFullCode As String) As String

'Converts an integer (from 0 to 10647) into a three digit/letter code. Letters are
in the sfullcode string.



    Dim CodeLen As Integer

    CodeLen = Len(sFullCode)


    If iNumber > CodeLen ^ 3 Then

        CalculateCode = "NULL"

        Exit Function

    End If


    Dim intPosition As Integer

    Dim intOstanek As Integer


    intPosition = Int(iNumber / (CodeLen ^ 2))
```

```
    intOstanek = iNumber Mod (CodeLen ^ 2)
    CalculateCode = Mid(sFullCode, intPosition + 1, 1)

    intPosition = Int(intOstanek / (CodeLen))
    intOstanek = intOstanek Mod (CodeLen)
    CalculateCode = CalculateCode & Mid(sFullCode, intPosition + 1, 1)

    intPosition = Int(intOstanek)
    CalculateCode = CalculateCode & Mid(sFullCode, intPosition + 1, 1)

End Function
```

In case of problems contact Tomaz Skrbinsek: tomaz.skrbinsek@gmail.com

7. ANNEX C

Table 5: Locus names, dyes, primer sequences and primer concentrations for the single-step multiplex PCR for genotyping of brown bear fecal samples in Slovenia.

Locus	5' primer	3' primer	Primer C [µM]	Allelic range
Mu10[B]	ATTCAGATTTCATCAGTTTGACA	6FAM-TCAGCATAGTTACACAAATCTCC	0.19	114-130
G10X[TP]	6FAM-CCCTGGTAACCACAAATCTCT	TCAGTTATCTGTGAAATCAAAA	0.40	132-154
G1D[P]	ATCTGTGGGTTTATAGGTTACA	6FAM-CTACTCTTCCTACTCTTTAAGAG	0.25	168-182
G10H[P]	6FAM-CAACAAGAAGACCACTGTAA	AGAGACCACCAAGTAGGATA	0.20	221-257
Mu50[B]	GTCTCTGTCATTTCCCCATC	6FAM-AACCTGGAACAAAAATTAACAC	0.06	79-103
G10P[T]	TACATAGGAGGAAGAAAGATGG	VIC-AAAAGGCCTAAGCTACATCG	0.09	122-150
Mu09[T]	AGCCACTTTGTAAGGAGTAGT	VIC-ATATAGCAGCATATTTTTGGCT	0.07	174-206
G10C[P]	VIC-AAAGCAGAAGGCCTTGATTTCCTG	GGGACATAAACACCGAGACAGC	0.05	97-116
SRY[B]	GAACGCATTCTTGGTGTGGTC	PET-TGATCTCTGAGTTTTGCATTTG	0.06	75
Mu15[T]	PET-CTGAATTATGCAATTAAACAGC	AAATAAGGGAGGCTTGGG T	0.15	117-131
G10L[B]	PET-ACTGATTTTATTCACATTTCCC	GATACAGAAACCTACCCATGCG	0.10	156-166
Mu59[B]	GCTCCTTTGGGACATTGTAA	NED-TGACTGTCACCAGCAGGAG	0.15	97-121
Mu23[B]	NED-TAGACCACCAAGGCATCAG	TTGCTTGCCTAGACCACC	0.07	142-156
[O] - (Ostrander *et al.*, 1993), [P] - (Paetkau *et al.*, 1998), [T] - (Taberlet *et al.*, 1997), [B] - (Bellemain and Taberlet, 2004)				

Table 6: Locus names, dyes, primer sequences and primer concentrations for analysis of brown bear tissues used in Slovenia.

Locus	5' primer	3' primer	Multiplex	C (µM)	Allelic range
G10C	5'-VIC-AAAGCAGAAGGCCTTGATTTCCTG-3'	5'-GGGACATAAACACCGAGACAGC-3'	A	0,07	89-109
G10D	5'-ATCTGTGGGTTTATAGGTTACA-3'	5'-6FAM-CTACTCTTCCTACTCTTTAAGAG-3	A	0,18	168-182

G10P	5'-TACATAGGAGGAAGAAAGATGG-3'	5'-VIC-AAAAGGCCTAAGCTACATCG-3'	A	0,09	147-175
G10X	5'-6FAM-CCCTGGTAACCACAAATCTCT-3'	5'-TCAGTTATCTGTGAAATCAAAA-3'	A	0,27	132-154
Mu10	5'-ATTCAGATTTCATCAGTTTGACA-3'	5'-6FAM-TCAGCATAGTTACACAAATCTCC-3'	A	0,16	112-126
Mu15	5'-PET-CTGAATTATGCAATTAAACAGC-3'	5'- AAATAAGGGAGGCTTGGG T-3'	A	0,25	117-131
Mu23	5'-NED-TAGACCACCAAGGCATCAG-3'	5'-TTGCTTGCCTAGACCACC-3'	A	0,11	142-156
Mu50	5'-GTCTCTGTCATTTCCCCATC-3'	5'-6FAM-AACCTGGAACAAAAATTAACAC-3'	A	0,10	79-103
Mu59	5'-GCTCCTTTGGGACATTGTAA-3'	5'-NED-TGACTGTCACCAGCAGGAG-3'	A	0,20	97-121
SRY	5'-GAACGCATTCTTGGTGTGGTC-3'	5'-PET-TGATCTCTGAGTTTTGCATTTG-3'	A	0,08	75
G10B	5'-GCCTTTTAATGTTCTGTTGAATTTG-3'	5'-6FAM-GACAAATCACAGAAACCTCCATCC-3'	C	0,10	130-154
G10H	5'-6FAM-CAACAAGAAGACCACTGTAA-3'	5'-AGAGACCACCAAGTAGGATA-3'	C	0,10	221-257
G10L	5'-PET-ACTGATTTTATTCACATTTCCC-3'	5'-GATACAGAAACCTACCCATGCG-3'	C	0,10	153-163
G1A	5'-VIC-GACCCTGCATACTCTCCTCTGATG-3'	5'-GCACTGTCCTGCGTAGAAGTGAC-3'	C	0,08	180-190
Mu05	5'-6FAM-AATCTTTTCACTTATGCCCA-3'	5'-GAAACTTGTTATGGGAACCA-3'	C	0,13	127-141
Mu11	5'-VIC-AAGTAATTGGTGAAATGACAGG-3'	5'-GAACCCTTCACCGAAAATC-3'	C	0,20	80-94
Mu26	5'-6FAM-GCCTCAAATGACAAGATTTC-3'	5'-TCAATTAAAATAGGAAGCAGC-3'	C	0,08	182-200
Mu51	5'-AGCCAGAATCCTAAGAGACCT-3'	5'-PET-AAAGAGAAGGGACAGGAGGTA-3'	C	0,09	115-127
Cxx20	5'-AGCAACCCCTCCCATTTACT-3'	5'-NED-TTGTCTGAATAGTCCTCTGCC-3'	D	0,30	121-141

G10J	5'-NED-GATCAGATATTTTCAGCTTT-3'	5'-AACCCCTCACACTCCACTTC-3'	D	0,10	78-97
G10M	5'-6FAM-TTCCCCTCATCGTAGGTTGTA-3'	5'-GATCATGTGTTTCCAAATAAT-3'	D	0,40	204-218
Mu09	5'-AGCCACTTTGTAAGGAGTAGT-3'	5'-VIC-ATATAGCAGCATATTTTTGGCT-3'	D	0,07	174-206
Mu61	5'-6FAM-TCCACTGGAGGGAAAATC-3'	5'-CTGCTACCTTTCATCAGCAT-3'	D	0,10	141-153

Table 7: Primers for amplification of microsatellite loci and for sex determination used in genetic research in Austria. Tann: annealing temperature; No. All.: Number of detected alleles. (References: a: Paetkau *et al.* 1995; b: Taberlet *et al.* 1997; c: Taberlet *et al.* 1993; d: Ennis and Gallagher 1994).

Locus	Primer sequence	Ref.	T_{ann}	No. All.
G10B	fwd: gcctttaatgttctgttgaatttg, rev: gacaaatcacagaaacctccatcc	a	56x2, 50x40	4
G1D	fwd: gatctgtgggtttataggttaca, rev: ctactcttcctactctttaagag	a	53x2, 47x45	4
G10L	fwd: gtactgatttaattcacatttccc, rev: gaagatacagaaacctacccatgc	a	56x2, 50x40	3
G10P	fwd: aggaggaagaaagatggaaaac, rev: tcatgtggggaaatactctgaa	a	53x2, 47x45	6
UarMU23	fwd: gcctgtgtgctattttatcc, rev: aatgggtttcttgtttaattac	b	53x2, 47x45	5
UarMU26	fwd: gcctcaaatgacaagatttc, rev: tcaattaaaataggaagcagc	b	53x2, 47x45	4
UarMU50	fwd: tctctgtcatttccccatc, rev: aaaggcaatgcagatattgt	b	53x2, 47x45	4
UarMU59	fwd: gctcctttgggacattgtaa, rev: gactgtcaccagcaggag	b	53x2, 47x45	5
SRY29, SRY121	fwd: aagcgacccatgaacgcatt, rev: gcttctgtaagcattttcca	c	50x50	1
SE47, SE48	fwd: cagccaaacctccctctgc, rev: cccgcttggtcttgtctgttg	d	55x50	2

8. ANNEX D

8.1. Fact sheets of projects involved in the genetic research of brown bears in the Alps - Dinara - Pindos and Carpathian Mountains

8.1.1. AUSTRIA

Title of project: "Genetic monitoring of brown bears in Austria"

Contact persons:

Georg Rauer, "Bärenanwalt", Research Institute of Wildlife Ecology (FIWI), University of Veterinary Medicine, Vienna, AUSTRIA

Elisabeth Haring & Luise Kruckenhauser, Laboratory of Molecular Systematics, Natural History Museum Vienna, Vienna, AUSTRIA

Study area: The Alps of Austria

The main focus so far was on the bear population that derived from the reintroduction project of WWF in central Austria. The migrants from the Trentino population to western Austria and a very few samples of the Carinthian bears have been analyzed, too.

General status of brown bears in the study area

The brown bear is protected year round by provincial hunting laws, in some provinces also by nature conservation laws. A management plan exists but is regarded only as a vague guideline by the responsible authorities. The EU Habitats Directive has not been fully implemented; Favourable Conservation Status of brown bears in Austria is not a relevant goal for the provincial governments.

Central Austria: one migrant from Slovenia settled in 1972; three bears were released in 1989-1993; 31 cubs were born in 1991 – 2006; largest population size 12 bears in 1999; today 2 male bears left; without restocking the population will cease to exist.
Carinthia: regular presence of single males; official number based on unpublished governmental data is 8 bears; no genetic monitoring.

Western Austria: sporadic migrants of the Trentino population since 2002.

Scientific status of the species in the study area:

Central Austria: monitoring of the development of the population since the start of the reintroduction project in 1989; conventional monitoring and radio tracking until 1999, genetic monitoring since 2000; 22 out of 35 known bears have been genotyped.

Carinthia: conventional monitoring.

Western Austria: conventional monitoring and genetic monitoring.

What is investigated and why genetics?

The aims are (1) to get a reliable estimate of bear numbers, (2) to determine the number of adult females in the population, (3) to detect events of immigration, (4) to draw a family tree, (5) to determine the level of heterozygosity, (6) to evaluate the risk of inbreeding, (7) to identify either dead or problem bears.

Bear numbers in central Austria were clearly overestimated by conventional monitoring. Genetic monitoring helped to reveal the exceptionally high mortality of sub adult bears and corroborated the speculation of illegal killings.

Project setup

2000 – 2007 the project was run by WWF, since 2008 it is run by FIWI

Field work: G. Rauer ("Bärenanwalt").

Analyzing lab: Molecular Systematics, Natural History Museum Vienna.

Finances: Ministry of Environment (2000-2001), EU Life Project (2002-2005), WWF (2005-2008), Freunde des Naturhistorischen Museums (2007), FIWI (2008-2009).

Field methods

Opportunistic sampling: collecting hairs and scats whenever encountered in conventional monitoring.

Hair traps: up to 27 hair traps in the core area of the bear population (500 km²); one strand of barbed wire 50 cm above ground around a bait; type of baits used: rotten fish intestines, blood, deer meat, or cattle intestines in different combinations presented in a bucket hung up between trees or poured on a heap of rotten wood (type of bait used actually: a bucket of corn hung up in a tree); controlling of hair

traps every 3 weeks; low visitation rate; roe deer feeding sites (wooden constructions where food is stored and offered) were the better hair traps, as these are regularly visited by bears; no mark recapture approach because of the low number of bears present, the aim was to detect, if not all, as many different bears as possible.

Lab methods

8 microsatellite loci (G10B, G1D, G10L, G10P, Mu23, Mu26, Mu50, Mu59) and 2 sex specific markers (SRY, SE) (Table 7, Annex C); PCR was carried out in a volume of 12.5 µl containing 10 mM Tris-HCl (pH 8.8), 1.5mM MgCl2, 150 mM KCl, 0.1 % Triton X-100, 0.25 units DynaZyme DNA-polymerase (Finnzymes OY), 4 pM of each primer (one primer labelled with IRD-800 or IRD-700), 200 µM of each dNTP, 0.25 µl DMSO, and 2 µl DNA solution. Annealing temperatures and number of amplification cycles are given in Table 7, Annex C. PCR products were separated in a LI-COR automatic sequencer. Analysis of PCR fragments was carried out with the software Saga Generation 2 and allele assignment was corrected manually.

Main results

22 bears fully genotyped; family tree constructed with the help of additional data of the conventional monitoring; several pairings between father and daughter detected; slight reduction in heterozygosity; clear indication of high mortality of sub adult bears found; no migration into the population; dispersal of sub adult bears followed; identification of dead bears (bone collected in the field and a sample taken from a dermoplastic).

Ongoing and planned future research activities

Continuation of the genetic monitoring in central Austria (as far as we know there are only 2 bears left but there are always rumours about more bears in the area; and there is a sharp debate about the need to restock); There is an urgent need to start genetic monitoring in Carinthia. We have no idea what's going on there, how many bears really live there and how long they stay (or live). The question of expansion of the Slovenian population into Austria and Italy is crucial for the plan of restoring the Alpine bear population and reach a Favourable Conservation Status.

Participation in a joint project

For Austria the main question is to understand the process at the northern fringe of the Slovenian bear population (expansion or no expansion). According to the

Habitats Directive the aim should be to promote or establish an Alpine bear population in a Favourable Conservation status. First step is to understand what's going on in the border area of Austria/Italy/Slovenia. Carinthia is somewhat reluctant to start genetic monitoring but in a cross border project we can motivate them to join in. In addition we expect along with the debate on the idea of restocking a growing interest of the EU into the bear situation not only in central Austria but also in Carinthia.

Document prepared by Georg Rauer, 8.5.2009

8.1.2. CROATIA

Title of project: "Genetic research of the brown bear in Croatia"

No current project; in the period 2006-2008 genetic research of bears was part of a larger project "Gaining and maintaining public acceptance of brown bear in Croatia" which is now finished.

Contact persons:

Đuro Huber; Ivna Kocijan (genetics)

Study area

The brown bear range in Croatia extends over 11,824 km2 and occupies the territory within the Dinara Mountain Range, connected with Slovenia on the northwest and Bosnia and Herzegovina on the east. Forest covers about 70% of habitat and is dominated by a mixture of beech, fir and spruce. Elevations range from sea level to 1,912 m with typical karst topographic features (limestone bedrock). The highest bear concentrations are in the regions of Gorski Kotar and central Lika (about 1 bear/10 km^2), in other areas densities are lower.

Tissue samples of hunted or traffic killed bears for our genetic research originate from the entire bear range, whereas scat samples originate from three study areas: two in Gorski Kotar and one in Velebit.

General status of brown bears in the study area

The brown bear is a game species in Croatia. The most important national legal provisions and documents which regulate bear management are the "Brown Bear Management Plan for the Republic of Croatia" (BMPC) (Huber *et al.*, 2008), the Hunting Act (Official Gazette 140/2005) and the Rules on Closed Hunting Season (O.G. 155/2005 and 48/2006). The bear hunting season is closed in the period May 1 – September 30 and December 16 – March 1, but exact dates are subject to change and the hunting season can be prolonged or shortened.

The Brown Bear Management Plan for the Republic of Croatia was first published in 2005 and the revised version in 2008 (Huber *et al.*, 2008). The BMPC summarizes international and national legal provisions relevant to bear conservation and management, the situation in Croatia and the management actions. On the annual

basis, decisions about hunting quotas and hunting season duration are brought in the Bear Action Plan.

The bear population is officially estimated at 600-1000 bears (Huber *et al.*, 2008), however the most recent unofficial estimate is near the upper side of this range and is probably reaching the carrying capacity and the social capacity of the habitat.

Main threats: hunting influence on sex and age structure, garbage conditioning and habituation to people and habitat fragmentation.

Scientific status of the species in the study area

There has been a considerable amount of research in bear biology and ecology, including home ranges and movements, habitat quality, body mass and growth, litter sizes, food habits, mortality causes, hematology and certain pathogens (serologic survey of viral and rickettsial agents and leptospirae). Genetic research has also been done (Kocijan, 2009).

What is investigated and why genetics?

We have decided to use genetic methods first as a way to estimate population size in Croatia. This approach was chosen as it was already being used in other populations (Scandinavian bear population, by Eve Bellemain) and considered more advantageous over field methods. No sound scientific method has been performed to estimate Croatian bear population before and our investigation was supposed to provide scientific data for population management (hunting quotas). About the same time we have started receiving tissue samples of hunted bears (legal obligation for hunters to send samples came into force in 2005), therefore we have decided to do analysis of genetic diversity as well. We used microsatellite loci and mtDNA control region sequencing.

Project setup

The project encompassed a human dimension study and a genetic study. It was financed by the MATRA Programme of the Dutch Ministry of Foreign Affairs. The total project budget was 80.000 EUR, half of which was intended for genetics (40,000 EUR). Croatian Ministry of regional development and forestry contributed by providing ca 20,000 EUR. Participating organizations were:

Veterinary Faculty (Đuro Huber and his team), Faculty of Science (Ivna Kocijan and Ana Galov), the Dutch NGO "Alertis" and about two dozen rangers who collected scat samples in the field. The project duration was two years.

Field methods

Scat samples were used for population size estimate and tissue samples for genetic diversity indices. Both types of samples were kept in 96% ethanol at ambient temperature and at 4°C upon receipt in the lab. Scat samples were collected in three study areas (Gorski Kotar North, G.K. South and Velebit) over two year periods mostly by park rangers who received a description of the sampling protocol together with sampling tubes.

Lab methods

Tissue: DNA is extracted with a commercially available kit (Wizard genomic DNA Purification Kit, Promega), followed by PCR amplification of 13 microsatellite loci (Mu10, Mu23, Mu50, Mu51, Mu59, G1D, G10B, G10C, G10J, G10L, G10M, G10P, G10X) and sex specific SRY locus. We use multiplex PCR combining up to 3 loci (Qiagen Multiplex PCR Kit). Reaction volume is 10 µL, temperature profile: 15 min at 94°C; followed by 30 cycles: 30 s at 94°C, 90 s at 60°C and 60 s at 72°C; final extension 30 min at 60°C.

A part of mtDNA control region (ca 320 bp) is amplified and sequenced (primers by Taberlet and Bouvet 1994).

Scat: DNA extraction (QIAamp DNA Stool Mini Kit, Qiagen); afterwards checking for bear DNA content by PCR with a single primer pair (Mu51) and agarose gel electrophoresis. Negative samples are discarded and positive are genotyped at 6 loci and SRY in two PCR multiplex reactions (PCR 1- Mu23, Mu51, Mu59, G10L; PCR 2- Mu10, Mu50, SRY). We use a multitube approach and up to 8 PCR repetitions to obtain reliable genotypes. Reaction volume is 10 µL, temperature profile: 15 min at 94°C; followed by 45 cycles: 30 s at 94°C, 90 s at 60°C and 60 s at 72°C; final extension 10 min at 60°C. Products are resolved by capillary electrophoresis in ABI-3100.

Main results

Tissue: 159 tissue samples have been genotyped for genetic diversity analysis. Mean allele number is 7,46 (5-10 alleles per locus), there is no deviations from HW across

loci and no evidence of a recent bottleneck. Two mtDNA haplotypes were found in 71 sequenced samples with frequencies 0,82 and 0,18.

Scat: DNA was extracted from 547 scat samples out of which 328 gave good quality genotypes. Scat genotyping success rate was therefore 67%. $P_{id\text{-}Sib}$ for six microsatellite loci used in scat genotyping is 0,51%. Population size was estimated by rarefaction method which produced a very rough estimate, but combined with other available data, directed the final estimate at about 1000 living bears.

Ongoing and planned future research activities

Currently we have no funds for bear genetic research and therefore there is no ongoing project. The plan is to repeat the population size estimate with more intensive scat sample collection at limited plots.

The idea for a future study is to do genetic diversity and structure analysis of the entire Dinara population, preferably in a joint project (this has already been discussed with Slovenian colleagues).

Participation in a joint project

We are especially interested in questions such as the genetic structure of Dinaric population and the whole Alps-Dinaric-Pindos, sex structure, effective population size and gene flow, and their application in management of the brown bear.

We can perform genetic analysis of all samples originating from Croatia and provide help in the form of advice and/or laboratory experience to partner countries which do not have a DNA lab. We are experienced in doing DNA extraction from tissue and scat samples, PCR amplification, gel and capillary electrophoresis and genotyping. However, due to the fact that we do not have a genetic analyzer (ABI machine) and depend on collaboration, we are rather limited in the number of samples we can genotype or sequence in a period of time.

Document prepared by Ivna Kocijan

8.1.3. GREECE

Title of project: "The Southwestern Balkans bear Register"

Contact persons:

Project coordinator: Alexandros A. Karamanlidis

Analyzing lab: Wildlife Genetics International, Dr. David Paetkau, CANADA

University of Zvolen, Prof. L. Paule, SLOVAKIA

Finances: ARCTUROS (2003 – ongoing), Alertis project (2008 – 2009), IBA (2008).

Study area

The protocol for the systematic non-invasive genetic monitoring of brown bears in the southwestern Balkans was developed in a main study area in the Prefecture of Grevena, in the central part of the Pindos mountain range that extended over almost 630km^2. Major forest vegetation types consisted of oak (*Quercus sp*.) and black pine (*Pinus nigra*). The area is characterized by a mosaic of dense forest, openings, and small scale cultivations. Elevations were 500 – 2.200 m, and human activity at lower elevations was intensive. The main study area is located at the centre of the species western nucleus of distribution in Greece and is currently the site of the construction of two major highways. We applied the protocol on a wider geographical scale and inspected therefore areas of known and expected bear distribution within Greece, Albania and FYROM. Additional study areas within Greece (i.e. Florina, Grammos, Trikala, Askio) were selected that were located also within the western nucleus of the distribution of the species in the country but were separated by the main study area by potential barriers to the movement of the species such as major highways, human settlements, rivers, etc. The study area in Florina was located to the north of the main study area, was 1.017 km^2 large and human population was dense. Grammos is a high mountain massif to the northwest of the main study area; the study site there was 740 km^2 big. The study site in Trikala was 708 km^2 big and was separated by the main study site by a highway. The study site in Askio was 500 km^2 big and separated by the study site in Florina by a major highway. Study sites in Albania (496 km^2) and FYROM (5615 km^2) were selected in the southern parts of both countries, close to the borders with Greece, in order to collect information that could eventually be used in the transborder management and protection of the species. In 2009 genetic research of brown bears was initiated throughout Serbia.

General status of brown bears in the study area

Brown bears in all three countries studied are considered to be endangered and none of the populations is estimated to number more than 200 individuals. The species is legally protected throughout the area, but conservation, management and research activities in all three countries have not succeeded yet in providing effective and long-lasting protection for the species.

Scientific status of the species in the study area

Despite more than 20 years of conservation efforts in the country little is known on the biology and ecology of brown bears in Greece. Some information has been published on the dietary preferences of the species and its activity patterns; however little is known on the species population status, distribution and genetic composition.

What is investigated and why genetics?

Due to the conspicuous lack of information on the size of the brown bear population in Greece, we decided to use genetic methods primarily to gain information on the number of bears in our country. Due to the specificities of our project (i.e. sampling from fixed locations over a long sampling period that resulted in several violations of mark-recapture principles) this has proven extremely difficult; however, due to the large number of samples collected (> 2,500) and analyzed we have managed to examine population history, genetic diversity and population structure in our study area.

Project setup

The project was initiated by the greek NGO ARCTUROS that financed the pilot study (15,000 EUR) in the area of Grevena in northern Greece in 2003. Since then, genetic analysis was carried out within the framework of the programme "Monitoring and evaluation of the effects on large mammals and their habitat due to the construction of the Egnatia highway in the connection area of the section "Panagia – Grevena" (Section 4.1 of the Egnatia highway connecting to the Central Greek highway" (20,000 EUR) and the project received financial assistance through a grant from the Dutch NGO "Alertis" (15,000) and the International Bear Association (1,700 EUR). Currently, field work in Greece is carried out by a biologist and volunteers of ARCTUROS, while in Albania, F.Y.R. Macedonia and Serbia field work is carried out

by members of the NGOs TWA, Mustela and the Macedonian Ecological Society respectively.

Field methods

In 2003 we recorded intense marking and rubbing behavior of brown bears on wooden poles of the electricity network (i.e. power poles) in Greece and used this behavior to develop a method for documenting the presence of the species in the country (Karamanlidis *et al.*, 2007). From 2003 to 2007 we inspected approximately 4500 poles throughout our study area and selected 350 of them to create a network of non-invasive sampling stations.

Power poles are made of wood that has been processed with a preservative (usually creosote) in order to resist damage caused by insects and rotting. Depending on land topography, poles are placed 50 – 100 m apart and the vegetation at a distance of 5m from each side of the pole line cleared away. Forest animals use these belts as travel corridors, with animal paths often leading from one pole to the next.

In order to minimize the chance of a bear rubbing against a pole without leaving hair behind and its visit going undetected, poles were fitted with barbed wires. A single piece of barbed wire was fitted to each pole, reaching from the ground to a height of approximately 2.0 m. Wraps around the pole were distanced approximately 30 cm from each other. From 2007 on, sampling stations have been visited on a monthly basis.

Lab methods

Genetic samples collected in the field were placed in paper envelopes without contacting human skin and then stored at room temperature in zip lock bags with silica gel (Roon *et al.*, 2003). For every collected hair sample, DNA extractions were performed using the DNeasy Blood & Tissue kits (QIAGEN, Hilden, Germany) following the manufacturer' s instructions. We aimed at using ten guard hairs where available. All extractions took place in a building in which amplified DNA had never been handled.

In order to test the polymorphism of genetic loci in the southwestern Balkans we screened 49 samples at the following 21 markers: *G1A, G1D, G10B, G10C, G10L, G10M, G10P, G10X* (Paetkau *et al.*, 1995), *G10H, G10J, G10O, G10U* (Paetkau *et al.*, 1998a), *CXX20, CXX110* (Ostrander *et al.*, 1993), *MU23, MU26, MU50, MU51, MU59*

(Taberlet *et al.*, 1997), *Msut-2* (Kitahara *et al.*, 2000), *REN145P07* (Breen *et al.*, 2001). Sex identification was established through the analysis of the amelogenin gene (Ennis & Gallagher, 1994).

Thermal cycling was performed using a MJ Research PTC100 thermocycler with 96 well 'Gold' blocks. PCR buffers and conditions were according to (Paetkau *et al.*, 1998a), except that markers were not co-amplified as co-amplification reduced success rates for hair samples. We used 3µl of a total extract volume of 125µl per PCR reaction, except during error-checking when 5µl was used. [MgCl2] was 2.0 mM for all markers except MU26 (1.5mM), MSUT-2 (1.5mM) and G10J (1.8mM). Microsatellite analysis used ABI's four color detection system; we used an automated sequencer (ABI 310) and genotypes were determined using ABI Genescan and Genotyper software. Error-checking and general quality assurance followed strictly the guidelines of (Paetkau, 2003).

Main results

The project is still ongoing and we have not had enough time to do a thorough analysis of the data. The results presented here are preliminary: We have identified so far 202 unique genotypes from 5 study areas in Greece (N = 195), in Albania (N = 2) and in F.YR. Macedonia (N = 5). The majority of individuals was identified in April, May and June and were males. The percentage of females rose to 40% during the autumn months.

Ongoing and planned future research activities

The project is currently in progress and funding has been secured until the end of 2009. Field work in Albania, F.Y.R. Macedonia and Serbia is currently supported by a conservation grant from Alertis. The cost of genetic research in these countries is now covered by the Greek NGO ARCTUROS. Field work and genetic research in Greece is supported by private funds from ARCTUROS. In 2010 the first genetic mark-recapture study in the western nucleus of the species in Greece is going to be carried out and samples from the eastern nucleus are going to be collected.

Ideally, the genetic data we have collected from the southern range of the Alps – Dinara - Pindos population would be compared with data from the northern range of the population in order to investigate genetic diversity and gene flow.

Participation in a joint project

We are especially interested in questions such as genetic structure of Alps-Dinaric-Pindos population, sex structure, effective population size and gene flow, and the application of such information in the conservation of the brown bear.

Document prepared by Alexandros A. Karamanlidis

8.1.4. ITALY - 1

Title of project: "Genetic monitoring of Central Alps brown bear population"

Contact persons:

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Study area: Central-Eastern Alps

The bears were introduced in the Adamello-Brenta Natural Park (PNAB), in the western part of Autonomous Province of Trento (PAT), but currently young males are ranging westward in Lombardia Region, eastward and southward in Veneto Region, and northward in Autonomous Province of Bolzano, Switzerland, Austria, and even one time in southern Germany, for a theoretical occupied area of ~17.000 km² in 2008. On the contrary, the core area, where females live, is much smaller (~1.160 km²) within the Trento Province.

The area is mainly mountainous. The altitudes in Trentino range from 67 m to 3.764 m. above sea level, with 27% of the area between 1,000 and 1,500 meters, 22% between 1,500 and 2,000, and 14% between 2,000 and 2,500 meters. Eco-climatic types range from alpine to sub-mediterranean. More than 50% of the 6,212 km² of the Trentino surface is covered by woods, even if interrupted by (sometimes highly) anthropized valleys, roads and infrastructures. It is worth to remember that the Alps are the most anthropized mountain range in the world, with a population of around 13,000,000 on a 190,912 km² surface (mean density: 60 inhabitants/km², which dramatically increases in tourist seasons).

General status of brown bears in the study area

The legal status of the brown bear follows directives and indications of EU in all countries interested by the presence of the species, except in Switzerland, outside EU, but maintaining strict contacts with other countries especially with the management of Autonomous Province of Trento.

The population was established from 10 individuals introduced in 1999-2002 in the PNAB, after the autochthonous population was declared biologically extinct (just 3-4

native bears remaining at the end of the nineties, old individuals, no reproduction registered since late eighties). Currently, no more native bears are present, and no participation of them to the reproduction with the introduced individuals was recorded. At the end of 2008, 24 (n. of unique genotypes found) to 28 bears are thought to be present.

The main task about the long-lasting existence of this population is related to Human Dimension, i.e. social acceptance of the species. The ecological suitability of the environment, estimated in 1997's feasibility study, is on the contrary well confirmed by the optimal reproduction rate and the general good physical conditions of all specimens which are captured, sighted, photographed or filmed.

One major threat is loss of individuals, many of them for unknown causes, which may be in some cases related to humans. Of the missing ones, three were found dead, four killed (legally in Germany and Switzerland or in accidents), one removed due to problematic behaviour and eight were not genetically sampled for at least two years. The population remains isolated.

Scientific status of the species in the study area

The brown bear population in the central Italian Alps is one of the scientifically best known in the world, because it was established by 10 introduced animals, and genetic samples were collected from 9 of the founders prior to their release, allowing determination of their genotype. Monitoring intensity has been very high, thanks to the involvement of a GO (Provincia Autonoma di Trento – Forest and Wildlife Service) since 1970s (traditional techniques such as radio tracking, VHF since 1976 + GPS since 2006; transects; snow tracking; camera traps; and genetic monitoring since 2002). Moreover, western Trentino is the only area in the Italian Alps where the brown bear never disappeared completely, so studies were carried out even throughout the last decades, and books were edited on bears in Trentino. The scientific knowledge about brown bear and the environmental suitability of the study area for the species is well reviewed in the feasibility study produced 1997 by ISPRA (Istituto Superiore per la Protezione e Ricerca Ambientale, formerly: INFS, Istituto Nazionale per la Fauna Selvatica) as prerequisite for the reintroduction project. In addition, studies about the brown bear in Trentino were conducted by PNAB (e.g. diet, habitat use, use and availability of dens, etc…) and analyses of radiotelemetry data (GPS + VHF) by PAT and PNAB.

What is investigated and why genetics?

Genetic monitoring was judged the most useful tool to gain up-to-date information about the development of the newly re-introduced population because it provides insight on demography, trends, reproduction, fate of single individuals, distribution and movements, connectivity with neighbouring populations as well as on genetic changes. Some of this information, including the ability of constructing the wild pedigree of the population, cannot be obtained using traditional field based methods. In addition, fast-line analyses (when required) may provide useful information about the identity of single animals of interest (problematic behaviour, involved in crisis situations, or other…). The opportunity of collecting genetic samples from the founders gives more power to the tool. In addition, costs and logistics can be reduced through careful sampling design and by opportunistic sampling.

Project setup

The genetic monitoring project on brown bear in Trento province started in 2002. It is coordinated by PAT with technical and scientific support of ISPRA. Field work is carried out by PAT and PNAB. The project is funded by PAT with recent support of Italian Environment Ministry. People involved are about 40/year, the personnel is trained by ISPRA and PAT, the duration of the project is unlimited. Following a decision taken in 2006, systematic collecting of samples with hair trapping is performed every second year (even years).

Field methods

Opportunistic sampling: incidental collection of hair and faeces was carried out throughout the bear range and during the whole year. Bear samples obtained from damages caused by bears were also stored for analysis.

Hair traps: We followed design outlined in previous DNA-based inventories in North America, and used a systematic grid. Cell size was small (4x4 km) in consideration of the topography of the habitat and human presence. Grid extent varied from 272 km^2 to 976 km^2.

Hair samples were stored in silica desiccant or in 95% ethanol; homogenized faecal samples of ~ 10 ml were stored in 95% ethanol. In 2008 we collected from the outside surface of the scat rather than homogenizing and obtained ~ 20% higher success rate than previous years. We compared success rates of scat stored in ethanol and in DET

buffer (Frantzen et al. 1998). Scat stored in DET resulted in ~5% higher success rates, however the use of DET is logistically difficult for our contest.

Lab methods

Extraction: Hair: Qiagen tissue kit; Feces Qiamp DNA stool kit (Qiagen Inc., Valencia, CA).

Microsatellite genotyping: 10 loci (G10X, G1D, G10M, G10P, Mu11, Mu15, Mu23, Mu50, Mu59, cxx20).

Sex ID: Amelogenin system.

PCR: Qiagen Multiplex PCR Kit; 2 multiplex (5 loci each); primer concentration 0.04-0.15 μM; touchdown PCR; 57.3° -52.5° annealing, 30 min. elongation at 60°.

Main results

Using genetic monitoring since 2002 we documented that the population increased from the initial 10 founders to at least 24 individuals (unique genotypes) at the end of 2008. The pedigree of the population obtained from genetic data allowed deriving detailed demographic information including number of individuals present each year, number of cubs (at least 35, in 16 litters) and individual reproducing, age of first reproduction of both sexes, inter-birth interval, and survival and growth rates. Genetic diversity is currently high (He= 0.74) but declining, inbreeding occurred, the level of relatedness in the population increased and no immigrants were detected. As a consequence effective population size (Ne) remained extremely small. Distribution of bear sample locations showed that the population is expanding into the former bear habitat beyond the surrounding of the translocation site. Young males are responsible for the largest movements from the natal area. All females as well as the two of founder males were always sampled in the core area.

Ongoing and planned future research activities

We plan to continue genetic monitoring to collect demographic, spatial and genetic data on the population with an increased participation and active involvement of other provinces and regions where the bear population is currently expanding. We want to use genetic monitoring to collect the comprehensive data necessary to gain understanding of the factors and processes influencing the dynamic and evolution of

this small isolated population, for guiding future management and conservation decisions.

Participation in a joint project

Our interest in a joint project arises from the need of coping with the conservation issues for this population which are mainly related to its small size and isolation and to non-natural mortality. To maximize chances of connectivity with the neighbouring populations of Slovenia and Austria, strong GO leadership is needed as well as long term financing of monitoring activities and inter-country coordination. We are willing to provide to the joint project the field and laboratory experience we gained in 7 years of genetic monitoring.

Document prepared by Davide Dalpiaz

8.1.5. ITALY - 2

Title of project: "Genetic monitoring of large carnivores in transboundary areas of Friuli Venezia Giulia - sustainable transboundary management of wildlife"

Contact persons:

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Study area: Central-Eastern Alps

The Friuli venezia giulia is located in the north east of Italy. The area is characterized by the alpine areas (2500 m above see, with a continental and alpine vegetation) and prealpine areas (continental vegetation) and karstic areas (mediterrenean vegetation), all of them with a dramatic increasing of forested areas.

General status of brown bears in the study area

The bear appears in Friuli Venezia Giulia region in the end of the 60th years coming from Slovenia. The most extended and apparent abundant presence of brown presence was verified from 1993 to 2000, and seemed correlated with the dynamics of the Slovenian population, at that time increasing. The estimation of the size of population was in the end of the 2000 of around 15 individuals, almost males. The presence in Friuli seems associated with a dispersion of male bears in relation to the presaturative condition in the core population in Slovenia. After 2000 the signs of presence and distribution seem to reduce with a dropping of the number of signs, in coincidence with the increasing of the hunting plane in Slovenia. Until 2004, the only information of the bear presence in our region came from the cropping and geographic elaboration of the sign of presence (tracks, depredation, hair and scats). No genetic analysis on the hairs and scats cropped were done until 2004.

Scientific status of the species in the study area

The presence of the brown bear until 2004 in Friuli Venezia Giulia was difficult to define in terms of number, sex ratio and use of habitat. The use of opportunistic signs have described only the distribution of bears. The combination of genetic and radiotelemetry after 2004 have permitted to observe as the presence for the brown bear in Friuli is seasonal, and only few individuals have as main territory the Friuli landscape. Some individuals spend only some months of its life in Friuli and then

return to the core areas in Slovenia, where are present females and feeding points. The absence of female doesn't permit to describe a real population but only the presence of peripheral population.

What is investigated and why genetics?

On the very small (sub)population is difficult to capture and monitored by radiotelemetry a significant number of individuals, especially when this sub population change in the time and in the space in terms of composition of individuals. The phototrapping doesn't permit to monitor the presence of occasional individuals, instead the genetics tracking permit to obtain information about the presence of different animals and for different period, during the different season and years. Genetics permits to identify the problematic bear. Genetic permits to study and identify a higher numbers of animals respect other techniques. Genetic permits to follow animals across different areas and different countries. Genetic permits to study the demographic trends and sex ratio. Genetic is a easy and efficient technique (especially for sampling) and is not invasive. The genetic can be integrated with phototrapping and radiotelemetry.

Project setup

The project has started in 2004 and has finished in 2007. The genetic project was a component of more complex project in which was utilized different techniques for the detection of he large carnivores as phototrapping, radiotelemetry and other methods useful for cropping opportunistic sign of presence (as snowtracking). The total amount of the project was 1.100.000 euro but for the genetic on the large carnivores (systematic+ opportunistic) was shared around 100.000 Euros. For this part was dedicated a PhD student (with grant) for DNA analysis and, in the 4 years, 8 undergraduate students and technicians (3) and researcher (1) for the cropping samples and preanalysis and data base.

This amount (travel, salary and grant) has covered 4 years of monitoring, around 3500 controls at the hair traps, with around 1250 samples cropped. In general we can estimate for cropping a sample, with hair traps (with a efficiency of 25%), covering travel, salary or grant, a cost from 40 to 80 Euros, when the cropping is performed not by volunteers.

In adding to this amount the regional office has financed the DNA analysis in the national institute of wildlife of 200 samples (cropped for the large amount by our institution) for 10,000 euro.

Field methods

We have established a network of hair traps (120) distributed along line transect (3 to 5 per line transect), these transect are choice on the basis of knowledge of expert. The hair traps, with barbed wires at 50 cm above ground, area baited, with corn and fish at the ground and on the hairs, and controlled every 15 days from March to November. The bear can eat the bait; this approach can induce a conditional behavior. The hair traps (120 hair traps) have covered 75000 (75 quadrants) hectares, with a core areas of 40000 hectares (70 hair traps). The hair traps collected are preanalysed (by microscope) to identify the species and then stored in the dry place with a paper envelope.

Lab methods

Genotyping data were collected at 6 microsatellite loci combining a semi-nested PCR with a multiplex preamplification method in order to increase the quality of the amplified DNA fragments and to avoid genotyping errors associated with low quantities of DNA. Allele number for each microsatellite locus ranges from 4 (UarMU10, UarMU51, G10L) to 7 (UarMu23, UarMU59). Individual identification was assessed for 10 brown bears ($Pid = 1.5 \times 10^{-6}$, $Pid_{sib} = 5 \times 10^{-3}$). Amelogenin gene analysis was used for sex identification and all genotyped animals resulted males. Multiplex preamplification method – multi tube approach. We have done 4 repeats for each sample and Genotype consensus accepted when PCR+ > 50%. In adding the national institute of wildlife - Randi added panel of 5 microsatellite loci: Mu15, G10P, G10M, G10C, G1D and have done analysis on some common samples.

Main results

The success of the hair traps change with areas and with the season, form 35% in spring to 26% in summer and 6% in fall; the number of hair of brown copped per hair traps was higher in April. (90 hairs as average) the success of genotyping for the opportunistic samples cropped from 1998-2003 was 23%, samples, with hair traps, 2004: 57%, samples 2005-2007, with hair traps: 73%; with a average of genotyping success rate: 68%. The Shannon index were: Mu59=1.822, G10M=1.680,

Mu50=1.657, Mu23=1.557, G1D=1.513, Mu15=1.431, G10C=1.375, G10P=1.373, Mu10=1.305, G10L=1.095, Mu51=1.072. Observed heterozygosis Mu59=0.700, G10M=0.700, Mu50=0.700, Mu23=0.700, G1D=0.700, Mu15=0.700, G10C=0.700, G10P=0.500, Mu10=0.750, G10L=0.250. We have indentified 10 different genotypes, from 2001 to 2006. All of them were males. During the systematic period (2004-2006) was detected an average of 5 different genotype per years. Allelic drop out: Mu59=0.009; G10M= 0.008, Mu50=0.011; Mu23=0.005, G1D=0.002, Mu15=0.000, G10C=0.013, G10P=0.077, Mu10=0.000, G10L=0.000, Mu51=0.003, AMG=0.050.

Ongoing and planned future research activities

We have only a small project the parco naturale delle prealpi giulie to monitor the presence of the large carnivores in the park but not specific on the large scale on the genetic. To give a continuity we have a genetic plan of research with a hair traps only in the core areas with only two controls planned.

Participation in a joint project

Is important to plan a project that permits to have a permanent system of genetic monitoring at transboundary level with a continuous system of exchange of data. Our institution can work on the samples cropping, with combination with other techniques as radiotelemetry and video trapping, and also in DNA analysis and on the creation of innovative integrated system of monitoring

Document prepared by Stefano Filacorda

8.1.6. SLOVENIA

Title of project:

1. Analysis of culled brown bears and molecular-genetic research of brown bear population in Slovenia (national-science, 2004-2007),

2. Conservation genetics and estimation of population size of brown bears in Slovenia (national-management, 2007-2008),

3. Hunting for sustainability (EU-FP7, 2009-2012),

4. Computational tools for conservation genetics and genetic monitoring of brown bear (national-science; 2009-2012)

Contact person:

Tomaz Skrbinšek

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Study area: Brown bears in Slovenia form the northwestern part of the Dinaric bear population

The area over which the bear is permanently present in Slovenia measures approximately 6000 km2. Most of the population lives in the S.E. part of the country, in the Dinaric Mountains – the core area. The core area covers approximately 4600 km2, and is directly connected to the rest of the Dinaric bear population over a long border with Croatia. A smaller number of bears live in the west of the country, where the Dinaric Mountains meet the Alps. These bears are separated from the rest of the population by the Ljubljana-Trieste highway, which is very difficult for the bears to cross. The size of this area is approximately 2000 km^2.

General status of brown bears in the study area

The brown bear is protected year round by the Decree of conservation/protection of threatened animal species. However, a yearly cull quota is set every year with the aim of regulation of the population size. The yearly bag differs between the years and has been between 70 – 120 individuals between 2000 and 2008. Using noninvasive genetic sampling and mark-recapture modeling we have estimated that the population size at the end of 2007, after the end of yearly cull and before reproduction, was 434 (394-475 95% CI) individuals. The expected spring numbers should exceed 500 bears.

Scientific status of the species in the study area:

The Slovenian part of the bear population has been well researched. Telemetry started in the 1990's, and is still continued with the use of GPS/GSM technology until the present day. While previously the focus was on basic brown bear ecology and was also used for habitat modeling, the focus of the recent studies is on bear-human interaction. Studies of the bear diet were done through examinations of digestive organs of killed bears and analysis of scats. Since 1995 all killed bears are examined, measured and tooth is extracted for age determination. This has been used to determine the age structure of the population.

Studies utilizing genetics have started in 2004 and are continuing to the present day. We have currently close to 700 tissue samples in our tissue bank. These samples have been used to determine the main genetic diversity indices, and are used constantly to monitor extraction of genetically tagged bears from the population. All bear mortality is routinely sampled since 2003 and genotyped using 22 microsatellite loci. Noninvasive genetics has been used in a pilot study to determine bear densities at two smaller areas (270 km^2 and 170 km^2, respectively) in 2004 and 2005. A large study using noninvasive genetics was done in 2007 and 2008 (sampling in autumn 2007) over the entire bear range in Slovenia, to provide a good estimate of the population size. Besides providing that, the study genetically tagged approximately ¾ of the bears present in the population at the time of the sampling, providing data for monitoring of the population in the future as well as for monitoring of transboundary movements of bears.

Scientific status – what is missing: We still lack a good human dimensions study. Population dynamics parameters should be better understood to enable modeling of population dynamics and better understand the impact of management actions. Population is very slowly expanding into the Alps, and we need to have a better, multidisciplinary understanding of the underlying causes. We need a better understanding of connectivity at the level of the entire Alps-Dinara-Pindos population to justify and promote management and conservation at the level of evolutionary significant units (ESU) and transcend the current country-oriented management.

What is investigated and why genetics?

For many of the questions and much of research mentioned above, genetics is currently the only tool able to provide reliable answers. This is the case for the population size estimates, but has also proven very effective in demonstrating the barrier effect of the Ljubljana-Trieste highway. Our studies using genetics, both using tissues and noninvasive samples, are evolving towards becoming a long-term population monitoring. We will continue sampling the bear mortality, and will (hopefully) do the next large-scale population survey in 2012. Also, we're currently working at using parentage and age data to establish reproductive characteristics of the population (reproductive output per age class and sex), and developing new methodology and software tools for matching of genotypes from error-prone samples and correction of the edge effect in mark-recapture studies, as well as software tools for efficient handling and analysis of large amounts of noninvasive genetics data we need for the long-term monitoring.

Project setup

We are financing most of our work through national funds, either science or management oriented. Some of our current research is financed through the 7th EU Framework Programme. The scope of financing is on average approximately 50,000 EUR per year.

One of the key components of our work is cooperation with Slovenia Forest Service and Slovenian Hunters Association. We have relied on them to provide field support for sample collection. So far, this has been done voluntarily and very efficiently. In all our research, we go out of our way to make cooperation as easy as possible for each participant. Also, we try to provide as much of feedback as possible through a number of channels to all the participants.

Main results:

Genetic diversity, bottleneck detection, population size / density estimates, barrier effect of the Ljubljana-Trieste highway

Ongoing and planned future research activities

Apart from continuing with the genetic monitoring, we would really like to expand the research in cooperation with the other countries from the region to promote management at the ESU level.

Participating in a joint project

We would like to understand the genetic substructure of the Alps-Dinara-Pindos population and how it relates with the underlying landscape, and try involving that knowledge in future management. Also, we'd like to participate in genetic tracking of bears across the region. We have a decently equipped laboratory, and can provide analysis of samples and/or training of graduate students (also possible for them to make a Ph.D. study at University of Ljubljana) from partner countries that could use the laboratory. We can help with organization of mark-recapture studies using noninvasive genetics and analysis of the data. We have significant GIS experience, and can participate with that in possible landscape genetics studies.

Document prepared by Tomaž Skrbinšek

8.1.7. SLOVAKIA

Title of project:

1. Genetic diversity and differentiation of wildlife populations (brown bear, lynx, chamois and red deer) (national-science, 2006–2009),

Contact person:

Ladislav Paule

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Study area: Brown bears in Slovakia and Romania

The area over which the bear is permanently present in Slovakia measures approximately 14000 km². Most of the population lives in the core area – Central Slovakia and smaller part of the brown bear population lives in the Eastern Slovakia, close to the Ukrainian and Polish border. Both Slovak subpopulations are from the Slovak side of the border not connected. The brown bear population is attached to the brown bear population in Southern Poland (approximately 80–100 individuals) and few individuals migrate to the Czech Republic. The Eastern Slovakian subpopulation is connected to the Ukraine.

General status of brown bears in the study area

The brown bear is protected year round by the Decree of conservation/protection of threatened animal species. However, a yearly cull quota is set every year with the aim of regulation of the population size. The yearly bag differs between the years and has been between 60–75 individuals between 2003 and 2008. The expert estimation of the brown bear population is about 700–800 individuals, although according to official statistics of game species (Ministry of Agriculture) there should be about 1,200 brown bears in Slovakia. Note: Ministry of Environment agrees with 700–800 individuals and from this populations size the annual bag is developed (10 %).

Scientific status of the species in the study area:

The Slovak part of the bear population has been well researched. In the period 1975-1980 two scientists Prof. J. Sládek (Faculty of Forestry, Zvolen) and Prof. P. Hell (Forest Research Institute in Zvolen) were dealing with brown bears. Prof. Sládek investigated craniometry, age structure of hunted brown bears, while Prof. Hell was dealing with ecology and management of brown bear populations.

Telemetry started in the 1998's, and is still continued with the use of GPS/GSM technology in the certain extent until the present day (Dr. S. Finďo). While previously the focus was on basic brown bear ecology and was also used for habitat modeling, the focus of the recent studies is on bear-human interaction. Studies of the bear diet were done through examinations of digestive organs of killed bears and analysis of scats. Since 1975 all killed bears are examined, measured and tooth is extracted for age determination. This has been used to determine the age structure of the population.

Studies utilizing genetics have started in 2005 and are continuing to the present day. We have currently close to 300 tissue samples in our tissue bank both from Slovakia and Romania. These samples have been used to determine the main genetic diversity indices. All bear mortality (in Slovakia) is routinely sampled since 2005 and genotyped using 13 microsatellite loci. Noninvasive genetics has been used in a pilot study in 2008. A large study using noninvasive genetics is planned to start in 2009 over the entire bear range in Slovenia, to provide a good estimate of the population size.

Scientific status – what is missing: We still lack a good human dimensions study. There were two NGO´s dealing with human – bear interactions, application of guarding dogs for management of farm animals. No systematic research has been done yet.

We need a better understanding of connectivity between Slovak and Polish and/or Slovak and Ukrainian brown bear population

What is investigated and why genetics?

The main aim of application of genetic methods to study brown bear populations within the above mentioned project was to compare population genetic parameters of the Slovak and Romanian brown bear populations – one originated from about 30 individuals remaining in between War period and the other one originated from about 800–1000 individuals (minimum number after the WWII).

Project setup

The project was financed by the national funds (300,000 EUR for three years for all four species). The project which should start later in 2009 should be financed from the Structural Funs of the EU (Ministry of Environment).

The key partner within the project was Slovak State Nature Conservancy which supplied all the samples of legally culled or accidentally killed brown bears. The Romanian samples were obtained by the cooperation with the ICAS and Faculty of Forestry in Brasov (Prof. O. Ionescu).

Field and laboratory methods: Tissue and faecal samples were stored in 96% ethanol, blood samples were stored with EDTA in –20 °C, bone and hair samples were stored in room temperature. DNA from tissues and blood was isolated either by modified method of phenol-chloroform extraction involving overnight digestion with proteinase K or by Chelex (Biorad). Bone samples were grinded at first, then decalcificated in EDTA several times and finally DNA was isolated by NucleoSpin Tissue Kit (Macherey-Nagel) according to manufacturer's protocol. Faecal and hair samples were processed in a laboratory for non-invasive samples. DNA from faeces was isolated by Qiagen Stool Mini Kit according producer's manual. Hair DNA was isolated by modified method of Chelex extraction (WALSH *et al.* 1991). Negative controls were used for detection of contamination.

Samples were genotyped using thirteen microsatellite loci originally published by PAETKAU & STROBECK (1994), PAETKAU *et al.* (1995) and TABERLET *et al.* (1997). Sex was determined by SRY locus (TABERLET *et al.* 1997). Some of primer sequences were redefined (BELLEMAIN & TABERLET 2004). Primers were amplified in three multiplexes: (1) Mu10, Mu23, Mu50, Mu51, Mu59, G10L, SRY; (2) G10B + G10C + G1D + G10P + G10X; (3) G10J + G10M.

Main results:

Genetic diversity, bottleneck detection,

Ongoing and planned future research activities

If the second project financed from the Structural Funds will start this autumn (the final decision is expected by the end of August 2009), the next two years will be devoted to the intensive work on sampling, genotyping and estimation of the population size. Further analyses will be done using the samples from Romanian Carpathians, aimed for better understanding the population structure within the range of Eastern Carpathians and, if possible, to fill the gap also with the Samples from Ukrainian Carpathians.

Further assistance in genotyping the samples from Greece. This activity has already been done in 2008.

Participating in a joint project

Our participation in a common project could aim at comparing brown bear populations originating from two different genetic lineages and elaborating sampling designs aimed at fragmented populations (landscape genetics). We have an equipped laboratory (including automated sequencer ABI 3130), and can provide training of graduate students (also possible for them to make a Ph.D. study at Technical University in Zvolen in the field of wildlife conservation genetics) from partner countries that could use the laboratory. If the larger project will start in 2009 the possibility to run analyses for partners in Zvolen will be limited but not excluded.

Document prepared by Ladislav Paule

Title of the project:

1. Behavioural ecology of red deer and brown bear in the Poľana mountains (national – management 2007–2009). Implemented by the National Forest Centre Zvolen
2. Methods of wildlife population estiamte and their aplicability for hunting management (national – management 2007–2012). Implemented by the National Forest Centre Zvolen
3. Brown bear corridors in Slovakia (international – management 2005 – ongoing). Implemented by the Carpathian Wildlife Society Zvolen

Contact person:

Slavomír Finďo

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Study area:

The area selected for studying bears within the projects no. 1 and no. 2 encompasses Veporské Mountains in central Slovakia including Poľana mountains destinctive by its volcanic origin. The study area spreads over approximately 50, 000 hectares and represents primary bear habitat. Mixed mountain forests composed mainly from spruce, beech and fir cover most of the area, while open landscape is dominated by farmland: meadows, pastures and areable land. An important land cover type is intersparesed woody vegetation that on many places creates corridors suitable for wildlife movement.

Basically the study area used for the project no. 3 covers the whole territory of Slovakia.

General status of the brown bear in study areas:

Brown bear is protected species and can be hunted only on exception issued by the Ministry of Environment after previous agreement with the Ministry of Agriculture, which is responsible for hunting management.

Scientific status of the species in the study area:

As far as it is known behavioural ecology of bruins has not been studied by modern methods. Thus there is actually no knowledge about home range size, movement,

habitat use and other biological features. Preliminary results have been achieved with regard feeding habits, body and skull parameters. First genetic study was done in the early 1990s. Up until the project n3 has been started, there was no knowledge in Slovakia about the impact of roads on big wildlife species.

What is investigated and why?

Within the projects no. 1 and no. 2 the main focus is to describe basic ecological parameters by use of VHF and GPS telemetry. Besides this we study food habitats of bears via scat analysis. Inter and intra species relationships on feeding stations have been investigated by use of stealth cameras. The project no. 3 deals with the identification of brown bear (alongside other target species) corridors across Slovakia intersecting main transportation network. We also deal with creation of habitat suitability model for bears.

Project setup

We are financing our projects mostly via funds provided by national organisations including the State Forests in Banská Bystrica and the Ministry of Agriculture in Bratislava. The project focused on road ecology is basically funded by the Frankfurt Zoological Society (Germany) and implemented by the Carpathian Wildlife Society (Slovakia).

Main results:

So far 3 bears have been collared, one with VHF and 2 by GPS techniques. The home range size ranged from 7,000 to 24,000 hectares. It was discovered that bears intensively use farmland especially maize fields and food provided by hunters to ungulates in form of supplementary winter-feeding. In spite of this fact there are no human habituated bears within the Veporské Mountains. We have just started in 2009 to study relationships of wildlife on feeding stations, thus so far no results are available. We identified relevant segments of major roads (highways and dual carriageways) across whole Slovakia intersected by bear corridors as well as by other big game species including red deer, wild boar, fallow deer, moufflon, wildcat, wolf and lynx. Identified neuralgic points has been considered by road building engineers as an important wildlife crossings where mitigation measures will be implemented e.g. green bridges.

Ongoing and planned future research activities

We plan to enlarge telemetry study of bears in 2009-2012 by use of GPS collars and to continue with studying feeding habits. It is planned to set up a doctoral study for the investigation of relationships of various wildlife species on feeding sites. The Ministry of agriculture insists on improvements of bear population estimate across the whole country as current practice produces completely unreliable results. Thus our focus will be to provide hunters with the methodology how to estimate bear population every year.

Participating in a joint project

So far not planned as no suitable projects were available.

Document prepared by Slavomír Finďo