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

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1. "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 "2nd International Workshop on the genetic study of the Alps – Dinara – Pindos and Carpathian brown bear (*Ursus arctos*) populations" 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 that have been 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.

1.1. 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 non-invasive 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.

1.2. 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.

1.2.1. 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 to consider. 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.

1.2.2. **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.

1.2.3. **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.

1.2.4. **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.

1.3. 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 1.6.1).

1.4. 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.

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.

1.4.1. **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.

1.4.2. **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 *et al.* 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 1).

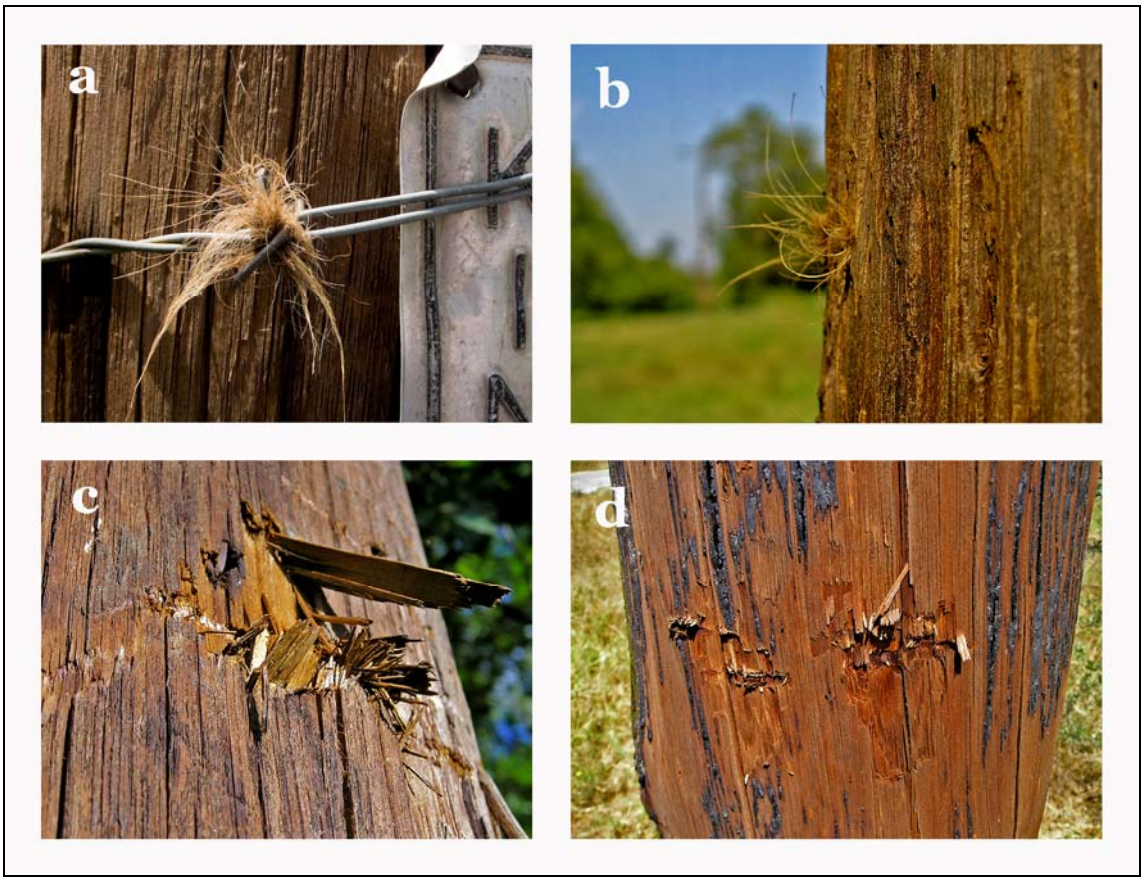

Figure 3: Deterioration rate of bear signs on power poles in the field in Greece (a: Stage 1 – Hair is long, curly and brownish, b: Stage 2 – Hair is short and blond, c: Stage 1: Big difference in colouration between newer and older marks and small pieces of wood sticking out of the pole, d: Small difference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

Table 1: 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.

1.4.3. **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 and 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.

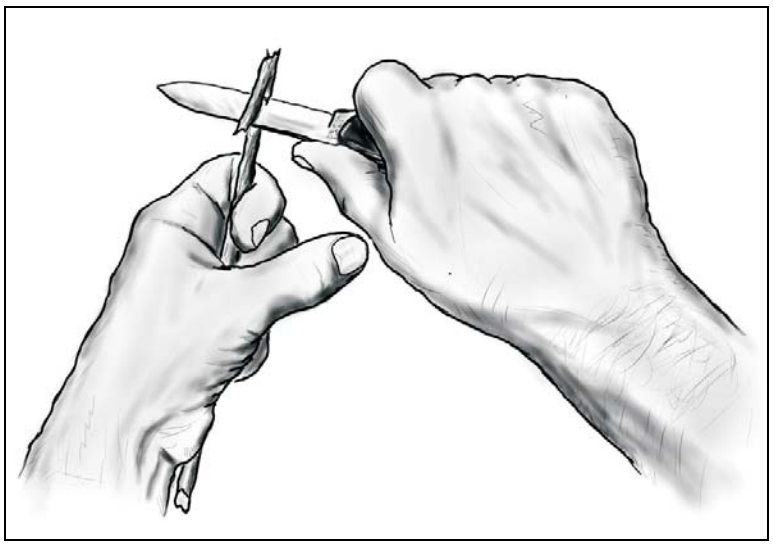

Figure 4: A good and simple tool for scat collection is a twig cut off flat on one side. After the collection, this "tool" is thrown away (© T. Skrbinšek).

- 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).

1.4.4. **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 1.2.2), 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 1.3) the sex of the animal and its estimated age and weight should also be recorded.

1.4.5. **Bone collection and storage**

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

1.5. 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.

1.5.1. 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 and Strobeck 2000, Poole *et al.* 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).

1.5.2. **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 2) 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 2: 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.

1.5.3. **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.

1.5.4. **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 are 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 and 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 and White 2009).

1.6. 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.

1.6.1. 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.

1.6.2. 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 the use of RfID chips, which are also becoming financially accessible.

1.6.3. **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.

1.6.4. **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.

1.6.5. **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.

1.7. 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.

1.7.1. 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.

1.7.2. 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.

1.7.3. 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.

1.7.4. **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.

1.7.5. **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.

1.8. 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.

1.8.1. Croatia

- **Tissue samples** were genotyped by amplifying 13 microsatellite loci [Mu10, Mu23, Mu50, Mu51, Mu59 (Taberlet *et al.* 1997), G10B, G1D, G10L (Paetkau and 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 *et al.* 2002).

1.8.2. **Greece**

In order to test the polymorphism of genetic loci in the southwestern Balkans 49 hair samples have been screened at 21 markers (Ostrander *et al.* 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).

1.8.3. **Slovenia**

The analysis protocol for scats is explained in detail in Skrbinšek *et al.* (in press). All 14 loci (Table 3, Annex B) 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 4, Annex B) 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.

1.9. Sex determination

It is possible to identify the sex of individual bears either through the analysis of the amelogenin gene (Ennis and Gallagher 1994) or the analysis of the SRY gene (Bellemain and 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.

1.10. 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 *et al.* 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 and 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.

1.11. 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 and Smouse 2006).
- Testing for evidence of recent bottlenecks events from allele frequency data can be achieved using BOTTLENECK v 1.2.02 (Piry *et al.* 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 *et al.* 2006).
- Testing for Hardy-Weinberg Equilibrium and LE can be achieved using GENEPOP (Raymond and 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 and Burnham 1999).
- Single-session population estimates from non-invasive genetic sampling data can be obtained with CAPWIRE (Miller *et al.* 2005).

1.12. 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

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 and Petit 2004), basic genetic diversity indices (Ho, He, A), probabilities of identity (Waits *et al.* 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 and 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.

2. 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 found in captivity.

3 LITERATURE CITED

ADAMS, J. R., and L. P. WAITS. 2007. An efficient method for screening faecal DNA genotypes and detecting new individuals and hybrids in the red wolf (*Canis rufus*) experimental population area. Conservation Genetics 8: 123-131.

BELLEMAIN, E., and P. TABERLET. 2004. Improved noninvasive genotyping method: application to brown bear (*Ursus arctos*) faeces. Molecular Ecology Notes 4: 519-522.

BOULANGER, J., G. C. WHITE, B. N. MCLELLAN, J. WOODS, M. PROCTOR and S. HIMMER. 2002. A meta-analysis of grizzly bear DNA mark-recapture projects in British Columbia, Canada. Ursus 13: 137-152.

BREEN, M., S. JOUQUAND, C. RENIER, C. S. MELLERSH, C. HITTE, N. G. HOLMES, A. CHERON, N. SUTER, F. VIGNAUX, A. E. BRISTOW, C. PRIAT, E. MCCANN, C. ANDRE, S. BOUNDY, P. GITSHAM, R. THOMAS, W. L. BRIDGE, H. F. SPRIGGS, E. J. RYDER, A. CURSON, J. SAMPSON, E. A. OSTRANDER, M. M. BINNS and F. GALIBERT. 2001. Chromosome-specific single-locus FISH probes allow anchorage of an 1800-marker integrated radiation-hybrid/linkage map of the domestic dog genome to all chromosomes. Genome Research 11: 1784-1795.

BROQUET, T., and E. PETIT. 2004. Quantifying genotyping errors in noninvasive population genetics. Molecular Ecology 13: 3601-3608.

BROQUET, T., N. MENARD and E. PETIT. 2007. Noninvasive population genetics: a review of sample source, diet, fragment length and microsatellite motif effects on amplification success and genotyping error rates. Conservation Genetics 8: 249-260.

COOCH, E., and G. C. WHITE. 2009. Program MARK, "A gentle introduction". http://www.phidot.org/software/mark/docs/book/

DE BARBA, M. 2009. Demographic and genetic monitoring of the translocated brown bear (*Ursus arctos*) population in the Italian Alps. PhD thesis, University of Idaho.

ENNIS, S., and T. GALLAGHER. 1994. PCR based sex determination assay in cattle based on bovine Amelogenin locus. Animal Genetics 25: 425-427.

FRANTZ, A. C., T. J. ROPER, L. C. POPE, P. J. CARPENTER, T. BURKE, G. J. WILSON and R. J. DELAHAY. 2003. Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using faecal DNA. Molecular Ecology 12: 1649-1661.

FRANTZEN, M. A. J., J. B. SILK, J. W. H. FERGUSON, R. K. WAYNE and M. H. KOHN. 1998. Empirical evaluation of preservation methods for faecal DNA. Molecular Ecology 7: 1423-1428.

GOUDET, J. 1995. FSTAT (version 1.2): a computer program to calculate F-statistics. Journal of Heredity 86: 485-486.

KALINOWSKI, S. T., M. A. SAWAYA and M. L. TAPER. 2006. Individual identification and distribution of genotypic differences between individuals. Journal of Wildlife Management 70: 1148-1150.

KARAMANLIDIS, A. A., D. YOULATOS, S. SGARDELIS and Z. SCOURAS. 2007. Using sign at power poles to document presence of bears in Greece. Ursus 18: 54-61.

KENDALL, K. C., J. B. STETZ, D. A. ROON, L. P. WAITS, J. BOULANGER and D. PAETKAU. 2008. Grizzly bear density in Glacier National Park, Montana. Journal of Wildlife Management 72: 1693-1705.

KITAHARA, E., Y. ISAGI, Y. ISHIBASHI and T. SAITOH. 2000. Polymorphic microsatellite DNA markers in the Asiatic black bear *Ursus thibetanus*. Molecular Ecology 9: 1661-1662.

MILLER, C. R., P. JOYCE and L. P. WAITS. 2002. Assessing allelic drop-out and genotype reliability using maximum likelihood. Genetics 160: 357-366.

MILLER, C. R., P. JOYCE and L. P. WAITS. 2005. A new method for estimating the size of small populations from genetic mark-recapture data. Molecular Ecology 14: 1991-2005.

MIQUEL, C., E. BELLEMAIN, J. POILLOT, J. BESSIERE, A. DURAND and P. TABERLET. 2006. Quality indexes to assess the reliability of genotypes in studies using noninvasive sampling and multiple-tube approach. Molecular Ecology Notes 6: 985-988.

MOWAT, G., and C. STROBECK. 2000. Estimating population size of grizzly bears using hair capture, DNA profiling, and mark-recapture analysis. Journal of Wildlife Management 64: 183-193.

MURPHY, M. A., L. P. WAITS, K. C. KENDALL, S. K. WASSER, J. A. HIGBEE and R. BOGDEN. 2002. An evaluation of long-term preservation methods for brown bear (*Ursus arctos*) faecal DNA samples. Conservation Genetics 3: 435-440.

MURPHY, M. A., K. C. KENDALL, A. ROBINSON and L. P. WAITS. 2006. The impact of time and field conditions on brown bear (*Ursus arctos*) feacal DNA amplification. Conservation Genetics.

MURPHY, M. A., K. C. KENDALL, A. ROBINSON and L. P. WAITS. 2007. The impact of time and field conditions on brown bear (*Ursus arctos*) faecal amplification. Conservation Genetics 8: 1219-1224.

OSTRANDER, E. A., G. F. J. SPRAGUE and J. RINE. 1993. Identification and characterization of dinucleotide repeat (CA)n markers for genetic mapping in dog. Genomics 16: 207-213.

PAETKAU, D., and C. STROBECK. 1994. Microsatellite analysis of genetic variation in black bear populations. Molecular Ecology 3: 489-495.

PAETKAU, D., W. CALVERT, I. STIRLING and C. STROBECK. 1995. Microsatellite analysis of population structure in Canadian polar bears. Molecular Ecology 4: 347-354.

PAETKAU, D., G. F. SHIELDS and C. STROBECK. 1998a. Gene flow between insular, coastal and interior populations of brown bears in Alaska. Molecular Ecology 7: 1283-1292.

PAETKAU, D., L. P. WAITS, P. L. CLARKSON, L. CRAIGHEAD, E. VYSE, R. WARD and C. STROBECK. 1998b. Variation in genetic diversity across the range of North American brown bears. Conservation Biology 12: 418-429.

PAETKAU, D. 2003. An empirical exploration of data quality in DNA-based population inventories. Molecular Ecology 12: 1375-1387.

PEAKALL, R., and P. E. SMOUSE. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology 6: 288-295.

PIGGOTT, M. P., and A. C. TAYLOR. 2003. Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species. Australian Journal of Zoology 51: 341-355.

PIGGOTT, M. P. 2004. Effect of sample age and season of collection on the reliability of microsatellite genotyping of faecal DNA. Wildlife Research 31: 485-493.

PIRY, S., G. L. LUIKART and J. M. CORNUET. 1997. BOTTLENECK: a computer program for detecting recent reductions in the effective size using allele frequency data. Journal of Heredity 90: 502-503.

POMPANON, F., A. BONIN, E. BELLEMAIN and P. TABERLET. 2005. Genotyping errors: causes, consequences and solutions. Nature Reviews Genetics 6: 847-856.

POOLE, K. G., G. MOWAT and D. A. FEAR. 2001. DNA-based population estimate for grizzly bears *Ursus arctos* in northeastern British Columbia, Canada. Wildlife Biology 7: 105-115.

RAYMOND, M., and F. ROUSSET. 1995. GENEPOP (version 3.3); population genetics software for exact tests and ecumenicism. The Journal of Heredity 86: 248-249.

ROON, D. A., L. P. WAITS and K. C. KENDALL. 2003. A quantitative evaluation of two methods for preserving hair samples. Molecular Ecology Notes 3: 163-166.

SOLDBERG, K. H., E. BELLEMAIN, O.-M. DRAGESET, P. TABERLET and J. E. SWENSON. 2006. An evaluation of field and non-invasive genetic methods to estimate brown bear (*Ursus arctos*) population size. Biological Conservation 128: 158-168.

STENGLEIN, J. L., M. DE BARBA, D. E. AUSBAND and L. P. WAITS. In press. Impacts of sampling location within a faeces on DNA quality in two carnivore species. Molecular Ecology Resources.

TABERLET, P., H. MATTOCK, C. DUBOIS-PAGANON and J. BOUVET. 1993. Sexing free-ranging brown bears *Ursus arctos* using hairs found in the field. Molecular Ecology 2: 399-403.

TABERLET, P., S. GRIFFIN, B. GOOSSENS, S. QUESTIAU, V. MANCEAU, N. ESCARAVAGE, L. P. WAITS and J. BOUVET. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Research 24: 3189-3194.

TABERLET, P., J.-J. CAMARRA, S. GRIFFIN, E. UHRES, O. HANOTTE, L. P. WAITS, C. DUBOIS-PAGANON, T. BURKE and J. BOUVET. 1997. Noninvasive genetic tracking of the endangered Pyrenean brown bear population. Molecular Ecology 6: 869-876.

VALIERE, N. 2002. GIMLET: a computer program for analysing genetic individual identification data. Molecular Ecology Notes 2: 377-379.

WAITS, L. P., G. LUIKART and P. TABERLET. 2001. Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. Molecular Ecology 10: 249-256.

WHITE, G. C., and K. P. BURNHAM. 1999. Program MARK: survival estimation from populations of marked animals. Bird Study (Supplement) 46: 120-138.

WOODS, J. G., D. PAETKAU, D. LEWIS, B. N. MCLELLAN, M. PROCTOR and C. STROBECK. 1999. Genetic tagging of free-ranging black and brown bears. Wildlife Society Bulletin 27: 616-627.

4. ANNEX A

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

5. ANNEX B

Table 3: 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 4: 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 5: 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