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"Coordinated Actions to Improve Wolf-Human Coexistence
at the Alpine Population Level"

Action A5-A6

Technical Report

INTERNATIONAL GENETIC PROTOCOLS FOR COST-EFFECTIVE MONITORING OF THE ALPINE WOLF POPULATION AND DETECTION OF HYBRIDIZATION

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We thank all the speakers and participants contributing to the LIFE WolfAlps EU Genetic Workshop on November 5th, 2020 for sharing their research, ideas, and suggestions to advance international wolf genetic monitoring in the LIFE WolfAlps EU project area and beyond. This technical report is based on the presentations and outputs of the Workshop. The names of all contributors are listed under the Genetic Wolf Alpine Group (GWAG) presented on page 4 of this document.

Summary of contents

Introduction and objectives of the document……... 3
The Genetic Wolf Alpine Group (GWAG)..... 4
Chapter 1 - Marker calibration among labs with standard microsatellites……..... 5
Chapter 2 - Strategy for Harmonization of »NEW« Genetic Markers in the Alps ……..... 8
Chapter 3 - A common international genetic agreement for detection of hybrids in the Alps..9
Chapter 4 - Public outreach and communication about hybridization…….....12
Glossary 14
Literature cited ……15
Appendix 1 - Microsatellite genetic markers used by different genetic laboratories ...……16

Introduction and objectives of the document

Prepared by Astrid V. Stronen and Francesca Marucco

The LIFE WolfAlps EU project seeks to advance coexistence between humans and wolves (*Canis lupus*) in the Alpine region, where a central objective is to develop coordinated and efficient population genetic monitoring across the project area. To achieve this objective, we are working on the development of shared genetic tools and approaches among the various countries involved. The online international genetic workshop organized by LIFE WolfAlps EU on November 5th, 2020, in the framework of Actions A5-A6, provided an update on current efforts and a forum to discuss long-term plans and goals, with focus on four central themes:

(1) Coordinating use and calibration of genetic markers across labs in the project area;
(2) Harmonizing new genetic markers and providing an update on work-in-progress;
(3) Developing an international genetic agreement and protocol for detection of hybrids, and
(4) Communicating with the public about hybridization and its implications for conservation.

The need for calibration of genetic profiles produced in different laboratories has long been a barrier to integration of data across management entities and genetic monitoring of transboundary populations. Such calibration is often costly and time-consuming, and requires considerable efforts while delaying efficient data sharing and potentially also management decisions. The issue is especially problematic for species such as large carnivores, which are wide-ranging and occur at low densities.

An important aim of the genetic work done in the framework of the LIFE WolfAlps EU project is therefore to **(A) coordinate genetic analyses** to ensure that different countries and laboratories analyse the same genetic markers, and to **(B) share reference samples for calibration among countries**, producing a reference data set of wolf profiles among participating laboratories. The first two chapters of this report summarize these efforts, which have greatly facilitated the ability to detect transboundary wolf packs. Detection of transboundary packs and their spatio-temporal distribution is fundamental to minimize double counts during evaluation of the Alpine wolf population and its conservation status, and permits identification of dispersing wolves across international borders.

Another major aim of the LIFE WolfAlps EU monitoring efforts is to ensure that we can distinguish wolves dispersing between genetically distinct populations (e.g., an immigrant from the Italian Alpine region into the Central European wolf population), from hybrids between wolves and domestic dogs (*C. l. familiaris*) and their descendants. After hybridization, standard genetic methods can usually detect the first-generation offspring (F1-hybrids) quite easily, also from non-invasive samples, because such genetic profiles will show equal contributions from each of the parent taxa (here, wolf and dog). However, detection of back-crosses to parent taxa becomes increasingly difficult with each new generation (e.g., Caniglia et al. 2020).

Gene flow among long-isolated wolf populations is considered beneficial for conservation, whereas anthropogenic hybridization is a long-term concern (Donfrancesco et al. 2019; Salvatori et al. 2020). Although wolf-dog hybridization has so far been rare within the project area, rapid and reliable detection of hybrids is essential. One important reason is that dispersing wolves from neighbouring populations and wolf-dog hybrids—all with genetic profiles divergent from the local Alpine wolf population—can occur in the project area simultaneously.

Hence, the two final chapters of this report address **(C**) the technical aspects of **monitoring and detecting hybridization** across the LIFE WolfAlps EU project area and relevant neighbouring regions, and **(D)** how we can **communicate with the public** in a clear and transparent manner about this complex topic. The last issue addresses current scientific knowledge and the complex (technical, legal, ethical) conservation challenges surrounding wild-domestic hybridization in general and wolf-dog admixture in particular. Anthropogenic hybridization in wide-ranging species such as wolves therefore illustrates the importance of international collaboration in promoting and achieving rapid, reliable, and transparent genetic monitoring approaches to support conservation management.

The Genetic Wolf Alpine Group (GWAG)

In the framework of the ***Wolf Alpine Group - WAG*** (WAG 2015), we have since 2001 been organizing a series of international meetings among wolf biologists and geneticists with the aim of standardizing and integrating wolf monitoring and genetic data for the Alpine wolf population. The genetic workshop on November 5th, 2020 was an opportunity to add new members to the overall group. The membership enlargement also reflects the fact that the Alpine wolf population is expanding and becoming increasingly connected to populations in neighbouring regions. Enlargement of the original group of specialists will therefore help facilitate the international monitoring efforts. The group that participated in the LIFE WolfAlps EU international genetic workshop and contributed to this document, and that will be in close cooperation with the Wolf Monitoring Group of the WAG in the framework of LIFE WolfAlps EU, is currently composed by:

- Geneticists from several countries and genetic labs that are members of the GWAG, as part of the WAG, are:
  SLOVENIA: Tomaž Skrbinšek, Marta De Barba, and Astrid V. Stronen (University of Ljubljana - Department of Biology - LIFE Project Partner);
  ITALY: Romolo Caniglia, Elena Fabbri, Edoardo Velli, Federica Mattucci and Nadia Mucci (ISPRA, Area per la Genetica della Conservazione (BIO-CGE); Mike Schwartz and Kristy Pilgrim from the National Genomics Center for Wildlife and Fish Conservation (NGC) in Missoula, Montana, USA, working with Italian Alpine samples since 2003 for the Large Carnivore Centre - Maritime Alps Protected Areas - LIFE Project Partner; Heidi Hauffe (Fondazione Edmund Mach); Pierluigi Acutis (IZS Torino), and Fabio Guglielmo (RAVA, laboratory of the Museum of Natural Sciences "Efisio Noussan" - LIFE project partner)
  FRANCE: Guillaume Queney and Cécile Kaerle (ANTAGENE) and Pierre Taberlet (CNRS, Laboratoire d'Ecologie Alpine)
  AUSTRIA: Steve Smith (University of Veterinary Medicine (VETMED), Konrad-Lorenz Institute of Ethology)
  GERMANY: Carsten Nowak (Senckenberg Research Institute and Natural History Museum Frankfurt, Wildlife Genetics Center)
  SWITZERLAND: Luca Fumagalli (University of Lausanne, Department of Ecology and Evolution), and Christine Breitenmoser (KORA)
  Other neighbouring countries: Pavel Hulva (Charles University, Department of Zoology (Czech Republic))

- The Wolf Monitoring Group of the WAG is composed of the following 1-2 representatives per Alpine country, and their membership in the GWAG is important for the broader connections and implications of the genetic strategies on the wolf monitoring programs. The WAG is composed by:
  SLOVENIA: Hubert Potočnik (University of Ljubljana - Department of Biology - LIFE Project Partner)

ITALY: Francesca Marucco (University of Turin, Department of Life Sciences and Systems Biology), and Elisa Avanzinelli (Maritime Alps Protected Areas - LIFE Project Partner)
FRANCE: Christophe Duchamp (French Biodiversity Agency (OFB) – LIFE Project Partner)
AUSTRIA: Georg Rauer and Felix Knauer (University of Veterinary Medicine Vienna (VETMED), – LIFE Project Partner)
GERMANY: Ilka Reinhardt (LUPUS German Institute for Wolf Monitoring and Research)
SWITZERLAND: Fridolin Zimmermann (KORA)

Chapter 1 - Marker calibration among labs with standard microsatellites

Prepared by Kristine L. Pilgrim and Michael K. Schwartz

Introduction

Molecular genetics has offered important insights into the life history, population dynamics, and behavior of wolves throughout Europe. Many different molecular genetic laboratories have played important roles in discovering this information about wolves at a local scale. The Wolf Alpine Group (WAG) first met in 2001 in Briancon (France) to begin to coordinate among different efforts in order to gain synergy among research groups and have the ability to make broader scale inferences. Eight subsequent meetings have led to broader coordination and concomitantly broader scopes of inference about wolf re-colonization of their historical range. These meetings were in Entracque (Italy) in 2004 and 2010, St. Martin France in 2005, La Fouly (Switzerland) in 2007, Jausiers (France) in 2013, Bormio (Italy) in 2015, and Podcerkev (Slovenia) in 2018.

After the 8th Wolf Alpine Group Workshop in 2015 the WAG agreed to 5 action items. These were to: **(1)** keep active communication among groups about the evolving new technologies, **(2)** exchange wolf tissue samples of previously genotyped samples for quality control and coordination among partners, **(3)** continue to exchange microsatellite genotype profiles among laboratories that are using a standardized set of microsatellites agreed to at an earlier WAG meeting in 2007, **(4)** test the High-Throughput sequencing (HTS) method for microsatellites developed by Pierre Taberlet's lab to ensure its transferability among different laboratories, and **(5)** test samples from across the wolf range with the Fluidigm approach to wolf genotyping developed at Carsten Nowak's group at the Senckenberg Research Institute (Harmoinen et al., in press).

The meeting on November 5th 2020, hosted virtually due to the global COVID-19 pandemic, further expanded this partnership under the umbrella of the Genetic Wolf Alpine Group (GWAG) and reported on the 5 action steps identified above.

List of tasks completed to date

The first workshop talk was by M. Schwartz and K. Pilgrim from the National Genomics Center for Wildlife and Fish Conservation (NGC) in Missoula, Montana. This talk summarized the work conducted between 2003-2014 and between 2015-2018 under the pre-LIFE WOLF project and in the LIFE WOLF ALPS project. In the pre-LIFE WOLF project 410 wolves were identified using 9 microsatellite loci all run on a traditional microsatellite

analysis platform. These samples were also run for sex identification and a mitochondrial D-loop haplotype analysis.

During the LIFE WOLF ALPS project 2,626 samples were analyzed identifying 468 unique wolves in the same area. The NGC also analyzed areas surrounding the initial study area between 2015-2018 and identified 614 individuals. All unique individuals across areas and time are genotyped at 16 microsatellite (also called STRs) markers, 4 y-linked microsatellite markers, and the K locus. **Appendix 1** provides an overview of the microsatellite markers used, and how they compare to other laboratories where standardization of microsatellite genotyping has been done under task 1, 2, and 3 listed above.

Noted in their talk were the advantages and disadvantages of using the microsatellite panel that has been standardized. The advantages are that new samples analyzed with this panel can be compared to hundreds of known individuals; many labs have access to or are currently using the standardized loci; the approach can be used to analyze one or two samples at a time (useful for forensic or emergency applications), and the approach can be used to identify dog alleles, thus allowing identification of hybrids. Further advantages of microsatellite markers in general are their conformation to population genetics models, which are well studied, and the markers fit neutral assumptions well. Errors are known and quantifiable with microsatellites, especially in their use with scats and other non-invasive genetic samples. Finally, most platforms have no proprietary components.

The second talk was by Marta De Barba et al. on the high-throughput sequencing (HTS) of microsatellites (STRs), following De Barba et al. (2017). This approach is very appealing because it allows for automation in running the samples and calling the alleles, and standardization of alleles comes from absolute not relative scores achieved from the sequencing platforms. This approach has been developed substantially since the publication of De Barba et al. (2017) in that there is no longer a proprietary step in the process, and it can now be achieved in any laboratory with access to a high-throughput sequencer (which is nearly all laboratories or genomics cores worldwide).

The disadvantages of the approach are that tetramer loci (or larger segments of up to 14 repeats) are used in the analysis, whereas the microsatellites in **Appendix 1** are dimers and would not be appropriate to use with the HTS approach. This means that any future samples run with this method would not be comparable to past samples until all historical samples were run with the HTS panel of microsatellites. Another disadvantage is that this method is not suitable for small numbers of samples (such as for emergency, law enforcement, or forensic situations).

Originally the HTS microsatellite approach standardized 13 tetranucleotide markers. However, since the last meeting a standard bioinformatic pipeline for marker design was developed and 200 markers were selected. There is also a pipeline that was developed for genotyping these markers automatically. Thus far this approach has been used to examine 317 samples using 44 markers (in 1 multiplex) identifying 237 wolves and 64 dogs. There were 155 tissues analyzed and 146 non-invasive samples.

The third approach being considered is an analysis of Single Nucleotide Polymorphism (SNP) markers using a Fluidigm platform, which is being pioneered by C. Nowak's group at the Senckenberg Research Institute (Harmoinen et al., in press). This approach uses SNPs which individually are less informative than microsatellites but it is easier to develop and analyze many SNPs. The Fluidigm approach uses markers that can be optimized for hybrid recognition or for increased variability, thus with the power to detect individuals. The typical Fluidigm platform performs analysis using a microfluidic circuit analyzing 96 SNPs by 96 samples, although this can be altered depending on the needs of the analysis.

Currently C. Nowak and colleagues have a panel that uses 40 SNPs for individual identification, 24 for hybridization, 28 for population genetic testing, and 4 for sex determination in felids. They are establishing a similar panel for canids, but are being careful about testing them with different quality samples. Their results suggest that SNPs on the Fluidigm perform better than microsatellites for low quality samples. Most of the non-invasive samples from wolves have very high amplification success (Kraus et al. 2014, von Thaden et al. 2017). These authors showed that there was very high power to discriminate individuals and identify hybrids with this approach, better than with microsatellite panels.

In summary, SNPs are cost effective, have relatively high throughput, have good cross-laboratory comparability, are automated, and the data is absolute which makes the data easier to replicate with other methods in the future. The disadvantages of this approach are with discerning mixed samples and the difficulty in running forensic or single samples, as entire panels must be run at a time. In addition, wolf samples analyzed with the SNP panel are not comparable with those run with the microsatellite panels (either the traditional panel or the HTS panel).

List of future tasks

We came close to arriving at a possible solution to the multiple approaches used, and all labs have added their loci to **Appendix 1**. The consensus seemed to be that microsatellites will be continued to be used by the labs that are running them, and once we agree upon a standard panel between labs we can take the next step to determine the best way to standardize scores/genotypes between these labs. As we want standardization of genotypes, we would want to go off of scores from the lab with the most samples and individuals in their database. The discussion included whether it was necessary to even implement the standardization of panels between labs if, in the long-term, the plan is to switch to HTS (i.e., is it worth the time to implement this if we should be focusing solely on switching to HTS?).

Most of the labs seemed to believe that HTS and SNPs will be best for future analysis. Many questions were posed on whether the primers/markers used for “standard” genotyping can be used in HTS analysis. More research and testing would be needed to confirm or deny if this is possible. Some labs could continue doing the “standard” genotyping where other labs would focus more on HTS and SNP implementation. A preliminary comparison of costs vs. timing for HTS sequencing and other methods is shown in **Table 1**. Lastly, there is a new approach that has been published by Eriksson et al. (2020) that uses high-throughput SNPs for canids and other carnivores. This approach has the benefits of each of the existing approaches and could be adapted for wolves in Europe.

Set of shared markers:

Appendix 1 shows the list of 16 microsatellites that are easily shared among labs, and includes the list of y and K-locus markers that can be readily shared with associated reference samples. All labs are willing to share their technology and samples, and the main issue is agreeing on a single approach with which to move forward. For the foreseeable future we will likely continue to use multiple approaches, which will be explored in Chapter 2.

Table 1. Initial overview of genetic approaches for wolf genetic analyses, including preliminary costs estimates, batch size, and the extent to which each method has been used to date. Methods for cost estimates have not been standardized so the estimated per-sample costs may not be directly comparable, but the table gives a general overview of the cost reduction the new methods provide.

	Microsatellite on Traditional Platforms	**HTS Microsatellites**	**SNP with Fluidigm**	**HTS SNPs**
Historical Samples Run	1000s	1000	1000	0
Minimum Batch Size	1 (48 preferred)	96 x 8 (preferred in one Illumina Miniseq run)	96	96
Cost per Batch	250-500€	850€	500-1000€	500€
Cost per Sample	50€	10€	8€	5€

Chapter 2 - Strategy for Harmonization of »NEW« Genetic Markers in the Alps

Prepared by Kristine L. Pilgrim, Michael K. Schwartz, and Tomaž Skrbinšek

Main objective(s):

Deciding on a common framework for analyzing samples with a shared approach.

List of tasks completed to date:

1) A table of common microsatellites among groups has been shared (Appendix 1);
2) A standard set of samples has been exchanged among groups.

List of future tasks:

1) All groups will continue to develop their respective approaches to meet their individual needs, and will also be able to compare new data to historical data;
2) The groups will continue to exchange samples and lists of microsatellites used;
3) All groups will share and train others, as needed, on their respective technologies;
4) The NGC (US) will explore the new SNP approach developed by Eriksson et al. (2020).
5) A new protocol for using high-throughput sequencing of microsatellites is in the final stages of development and will be finalized within LIFE WolfAlps EU (collaboration among University of Lausanne (CH), Alpine Ecology Laboratory (F), and University of Ljubljana (SLO)).

Additional comments/questions to resolve:

At some point in the future when technology allows us to produce genotypes at a reduced cost, it would be beneficial to run historical samples either with SNPs or with HTS microsatellites. The timeline and chosen technology will depend on the future development of each of these approaches, and testing of the SNP methods by Eriksson et al. (2020).

Chapter 3 - A common international genetic agreement for detection of hybrids in the Alps

Prepared by Romolo Caniglia, Elena Fabbri, Edoardo Velli, Federica Mattucci and Nadia Mucci

Main objective(s):

The main objective of this chapter is to define a common international and inter-lab genotyping protocol for reliable wolf-dog hybrid detection in the Alpine wolf population.

Introduction:

The correct identification of wolf-dog hybrids and introgressed individuals requires appropriate methods for genetic and statistical analyses, and representative reference populations in the assignment tests, to avoid erroneous or unreliable results. It has been demonstrated how certain laboratories without the required experience in wildlife genotyping or appropriate reference populations have produced unreliable results. This situation can lead to (1) overestimates of the number of individuals and (2) identification of false hybrids (i.e., when individuals are erroneously classified as hybrids).

It seems crucial for the project to (*i*) focus management attention and intervention actions toward recent hybrids (F1, F2, BC1, BC2) because they have the highest probabilities of distributing genes from domestic dogs into wolf populations, and to (*ii*) clearly distinguish genetic studies centered on solving important and urgent management and conservation problems from genetic research focused on improved understanding of hybridization in a long-term evolutionary perspective.

Methods employed:

Several analytical methodologies have been used or are under development in genetic monitoring projects to investigate the distribution of wolves and wolf-dog hybrids at the European level:

- i) classical STRs: different STR panels are applied in non-invasive wolf monitoring projects in Italy, France, Germany, Austria, Slovenia, and Switzerland;
- ii) SNPs: a 96-SNP panel was developed with Fluidigm methodology by Carsten Nowak's group (Harmoinen et al., in press) for wolf-dog hybrid detection at the European level. A 192-SNP panel selected by the ISPRA group (Italy) (Stronen et al., submitted) is under optimization for individual identification and to distinguish anthropogenic and natural hybridization (hybrids between wolves and dogs, vs. (potential) hybrids between wolves and golden jackals (*C. aureus*));

- iii) HTS-STRs in development by Luca Fumagalli, P. Taberlet, M. De Barba and collaborators (Switzerland, France, Slovenia) for individual and hybrid identification in the Alpine regions).

Brief description of methods used for rapid analyses of non-invasive samples

- **Germany**: A 13 STR set plus two sex markers are applied in legal wolf monitoring, supplemented by regular analysis of the 96-SNP panel (Harmoinen et al., in press) of selected samples for detection of hybridization.

- **Italy-ISPRA**: a 12-STR panel (plus a fragment of the hypervariable portion of the mtDNA D-loop and 4 Y-STRs) is currently applied for the genotyping of non-invasive DNA and a 39-STR panel (plus a fragment of the hypervariable portion of the mtDNA D-loop and 4 Y-STRs) for the genotyping of good quality DNA. An expanded 192-SNP panel has been selected for (*i*) identification of canid species (wolf, dog, golden jackal), (*ii*) wolf populations with distinct genetic profiles (Italy, Iberian Peninsula and the Dinaric region, and individuals with admixed ancestry from different wolf populations), (*iii*) identification of hybridization between naturally-occuring wild canids (wolf-golden jackal) or anthropogenic hybridization (wolf-dog; jackal-dog), and (*iv*) identification of individuals from non-invasive samples (low Probability of Identity (PID) and PIDsibs; Waits et al. 2001). The 192-SNP panel, which incorporates SNPs from the 96-SNP panel, will be tested on the Fluidigm system thanks to a collaboration with C. Nowak's group at the Senckenberg Research Institute in Germany.

- **Italian western and eastern Alps** (National Genomics Center (NGC) lab): ISPRA shared its own genotyping protocols with the NGC Lab, which will apply them for analyses of non-invasive samples collected during the monitoring activities for the LIFE WolfAlps EU project. Samples from the WolfAlps project area where canids show anomalous phenotypic characters (suspected hybrids) will be analysed by ISPRA for an in-depth investigation of possible hybridization and assignment tests.

- **Italian central Alps** (Fondazione Edmund Mach (FEM) lab): ISPRA shared its own protocols with the staff at FEM, who will apply them for non-invasive analysis during monitoring. However, samples from suspected hybrids will be analysed in collaboration with ISPRA, based on profiles from their reference populations.

- **France:** STR markers (22 loci), OFB-ANTAGENE

- **Switzerland**: (UNIL Lausanne): a 11-STR panel (plus a fragment of the hypervariable portion of the mtDNA D-loop and 1 Y-linked sequence) is currently applied for routine genotyping of non-invasive and tissue DNA. HTS of microsatellites is in the final development phase and will replace the former (see below description for Slovenia).

- **Slovenia**: For routine monitoring, a panel of 16 STR markers and a sex marker (amelogenin) is used for individual ID. Additionally, 8 STR markers and another sex marker (SRY) are analyzed for each recognized individual for pedigree reconstruction, species ID (canids) and hybridization detection. A high-throughput sequencing (HTS) approach is in the final stages of development, the plan is to start using it already in the analysis of the 2020-2021 sampling season, and to switch to the new protocol exclusively in the 2021-2022 season. With this new protocol the individual ID panel includes 22 STR markers and a sex marker, and the extended panel with an additional 22 STR markers can be used (if needed) for pedigree reconstruction and hybridization detection. We also started checking suspected

hybrids with the hybrid-detection SNP panel in collaboration with the Senckenberg Research Institute.

- **Austria**: at the moment, STRs and amylase gene copy numbers are used simultaneously because only a small amount of samples (< 400) are collected per year. In case of potential hybrid samples, they will collaborate with C. Nowak's group to genotype these individuals with the 96-SNP panel.

Proposals

- ISPRA: A shared STR panel or a shared SNP panel, or ideally a combination of the two methods. For example, non-invasive genetic monitoring projects could be performed using a common panel of microsatellites. Next, unique genotypes with good-quality DNA could be typed with SNPs, for improved wolf-dog hybrid detection.

- Sharing of reference samples:
  - ISPRA will collaborate at the Alpine scale in exchanging DNA from non-invasive and invasive samples, to perform analyses and test the applicability of new techniques such as HTS-STR and the 96-SNP Fluidigm panel.
  - Austria (University of Veterinary Medicine Vienna – VUW) will cooperate at the Alpine scale by exchanging individual data and haplotypes. They can send extracted DNA of all individuals identified in Austria that show Italian, Dinaric, or Carpathian haplotypes (20-30 per year) to the labs using the HTS approach. Conversely, data showing Central-European (CE) haplotypes will be shared within the CE-Wolf consortium. VUW will cover the costs of the analyses, and the results can be freely exchanged among labs.

Populations/reference samples needed:

- Selected wolf and dog reference populations (Italian, Dinaric, Balkan, and Central-European wolves; village and purebreed domestic dogs) are necessary to perform reliable assignment procedures: reference samples should have no morphological nor genetic anomalies (if possible determined from genomic profiles), and should as far as possible be representative of the genetic variability of the investigated populations.
- One option is an inter-laboratory exchange of analytical results (only *q*-values not genotypes). – ISPRA proposal.
- Alternatively, we could identify one reference conservation laboratory that performs the statistical analyses of genotypes for hybrid detection. – ISPRA proposal.

List of tasks completed to date:

- Reference individuals obtained for the Italian wolf population and domestic dogs (including village dogs and breeds of comparable wolf size and appearance).
- Reliable and robust Bayesian analysis protocols developed for hybrid detection using the ISPRA STR panel (Structure, Parallel Structure, $K = 2$ (Pritchard et al. 2000)).
- Shared protocols and list of markers currently used (NGC: done; FEM: done; Massimo Scandura & Marco Apollonio's lab, University of Sassari (Sardinia, Italy): done).
- Panel of 96 SNPs developed for detection of wolf-dog hybridization in Europe (Harmoinen et al., in press). The panel is suitable also for non-invasive DNA samples, and has been successfully tested for wolf populations across Europe.

List of future tasks:

- Synthetic DNA for use as an allelic ladder to calibrate allele sizes for the shared STR panel may be created in the near future.
- Sharing of the STR marker list and analytical protocols currently used by different conservation genetic labs should be completed in the near future.

What levels of hybrid detection (e.g., F1, BC1) can we reliably provide?

- The detection of hybrids at least until BC1 would be desirable and, at present, 100% of the F1-hybrids, 100% of the BC1-individuals, and 71%-93% of the BC2-individuals in the Italian population can be successfully identified using the ISPRA 39-STR panel.
- However; it would be necessary to carefully verify the rate of accurate hybrid detection for the Dinaric wolf population when applying traditional STRs or ISPRA STRs.
- Using the ISPRA 12-STR panel: 100% F1, 86%-94% of BC1W and 48%-60% of BC2W (depending on the *q*-thresholds applied) can be successfully identified.
- The 96-SNP panel (Harmoinen et al., in press) can reliably distinguish parental taxa (i.e., wolves and domestic dogs), F1-hybrids, and the first generation backcross to wolves (BC1). Additionally, second and third-generation backcrosses to wolves (BC2, BC3) can in most cases be identified as advanced hybrids.

Additional comments/questions to resolve:

In the near future, it would be helpful to verify the resolution of the ISPRA SNP panel for detection of BC2-individuals (i.e., the 2nd generation of backcrossing into parent taxa).

- Verify the resolution of the ISPRA STR panel for detection of hybrids and backcrosses (F1, BC1, BC2) in the Dinaric wolf population.
- Identify common and (relatively) reliable phenotypic characters as possible indicators of hybridization or back-crossing (introgression) into parent taxa. Although hybridization typically requires genetic confirmation because of the diverse expression of phenotypes, phenotypic traits could provide important information in combination with e.g., camera trapping for identification of target areas for non-invasive genetic monitoring.

Chapter 4 - Public outreach and communication about hybridization

Prepared by Astrid V. Stronen and Marjeta Konec

Before the final discussion on communication and outreach about hybridization, P. Taberlet presented key findings from a study that had provided DNA test samples for analyses at selected laboratories. The laboratory results were highly variable, and underlined the need to carefully consider the analytical context and methods used for species assignment and detection of potential hybrid individuals. Importantly, analyses of non-invasive samples must use current methods that include multiple replicates of each sample, to account for the risk of errors (allelic drop-out and false alleles) associated with low quantity and quality DNA.

Notably, the framework and standards of forensic analyses may not always align well with wildlife/conservation genetic analyses nor with subsequent communication about the results obtained. In particular, communication about results from putative hybrids done in the framework of forensic analyses may focus on not excluding any possible chance of hybridization, even if this probability is exceedingly low. However, such communications may risk being interpreted as statements confirming that hybrids have been identified. Furthermore, the probability of confounding results and interpretations will increase where genetic analyses have not included all relevant reference populations (Harmoinen et al., in press).

Consequently, internationally coordinated approaches to hybrid analyses that involve (*i*) methods agreed upon among countries and laboratories, (*ii*) rigorous assessment of non-invasive DNA, and (*iii*) shared samples from all relevant reference populations—determined by the geographic area of interest—is the recommended way forward to implement transparent, consistent, and reliable analyses of putative hybrids.

How can we communicate clear, accurate, and transparent results to the public?

DO

- It is important to meet with local people and explain new findings about hybridization, and a series of public meetings will often be necessary. However, this work is time consuming and may require extra staff/hours.

- The LIFE projects that include persons experienced in communication, social sciences, and genetics are very good opportunities for collaboration on public outreach, for example to prepare communication strategies in the event of hybridization issues.

- In addition to peer-reviewed (and ideally open-access) scientific papers for international journals, it is very important to make results and conclusions available in local languages, for example by submitting articles to national magazines for livestock breeders.

- An international standardized method used across different genetic labs and countries (and possibly also having one designated reference lab conducting this work) can help achieve consistent reporting of hybrids. As the public may, at times, have more trust in foreign labs, a standardized international approach is also helpful nationally, in case genetic labs experience that local interest groups have limited trust in their analyses and conclusions.

DON'T

- Although useful at times, working with local media (e.g., newspapers) and social media to distribute scientific results on hybrids may not always be feasible, because of local resistance and/or because keeping up with responses to social media posts is time-consuming for many scientists or organisations with limited staff. For example, it can be difficult to publish scientific results in local newspapers, including texts written for a general audience, as such reports may be deemed more 'boring' than unverified news about hybrids.

- Do not expect local stakeholders to be interested in specific methods and analytical details, so make sure to provide them with information that is not too technical (but that technical information can be offered if/where needed).

How can we communicate if we consider that a study reporting hybrids was NOT done in a clear, accurate and transparent manner?

DO

- Organised efforts to provide samples to different genetic labs for blind tests can provide objective and transparent reporting on their experience and levels of expertise in analysing hybrids in canids and other wild species.

- Based on such lab evaluations, local interest groups and government agencies that have earlier interacted with these labs may conclude that their performance can vary considerably, and that not all claims of hybrids are verifiable. Such claims may instead have been made because of (1) lack of proper reference populations and/or (2) the need to cover all options in the possible event of legal proceedings.

- It is important to communicate with livestock breeders about predation issues and provide feedback on their submitted samples and the analytical results. The information provided by different actors (e.g., different labs, agencies) to livestock breeders can be bewildering, and they may often feel that they are left alone.

- Definitions of 'hybrid' can vary widely, and efforts toward a shared terminology, e.g., how to categorize different levels of back-crosses, can help to mitigate this confusion.

DON'T

- Do not expect that commercial labs will want to share their methods or reference samples, or to be focused on/interested in transparent and open scientific research.

Glossary

BC1 – see F1
BC2 – see F1
F1 – first-generation hybrid, the offspring of reproduction between two different species, e.g., a wolf and a domestic dog. Subsequent reproduction events can then produce a F2-hybrid (offspring of F1 x F1), BC1 (back-cross to one parental group; F1 x wolf, or F1 x dog), BC2 (BC1 x wolf, or BC1 x dog), etc.
F2 – see F1
FEM – Fondazione Edmund Mach (IT)
GWAG – Genetic Wolf Alpine Group
HTS – high-throughput sequencing
ISPRA – Istituto Superiore per la Protezione e la Ricerca Ambientale (IT)
NGS – National Genomics Center for Wildlife and Fish Conservation (US)
Microsatellites – (also called STR) short repetitive DNA segments, usually 2-6 base pairs in length
SNP – single nucleotide polymorphism
STR – (also called microsatellites) short tandem repeats: short repetitive DNA segments,usually 2-6 base pairs in length
VUW – University of Veterinary Medicine Vienna (AT)
WAG – Wolf Alpine Group

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Appendix 1. Microsatellite genetic markers used by different genetic laboratories involved in LIFE WolfAlps EU. Y-chromosome markers are shown in bold font. Abbreviations for laboratory names and organizations are provided in the footnotes.

Locus	NGC	ISPRA	Senckenberg	Previously Standardized	VUW	UNIL Lausanne	UL Ljubljana	OFB-ANTAG ENE	RAVA Lab - Aosta Valley
CPH2	y	y			y		y	y	
CPH4	y	y			y		y		
CPH5	y	y	y	y	y	y	y	y	y
CPH8	y	y					y		
CPH12	y	y			y		y		
C09.250	y	y					y	y	y
C20.253	y	y					y		
FH2088	y	y	y	y	y	y	y	y	y
FH2096	y	y	y	y	y	y	y	y	y
FH2137	y	y	y	y	y	y	y	y	y
FH2004	y	y					y	y	
FH2079	y	y							
FH2140	y		y	y	y	y		y	y
FH2054	y		y	y	y	y	y	y	y
FH2161	y		y	y	y	y		y	y
Pez17	y		y	y	y	y		y	y
MSY34A	y	y							
MSY41A	y	y							
MSY34B	y	y							
MSY41B	y	y							
K-locus	y	y							
FH2001			y		y	y			
FH2010			y		y	y	y	y	
FH2848					y		y		
INRA21					y		y		
AHT137					y		y		
REN169D01					y		y		

PEZ01					y	y			
AHT103								y	
AHT111								y	
AHTk211							y	y	
C09.173								y	
CFX30371								y	
C22.279								y	
INU030							y	y	
C27.442								y	
Dbar1								y	
REN162C04							y	y	
AHTh171							y		
AHTh260							y		
AHTk253							y		
CPH22							y		
CPH6							y		
CPH7							y		
CPH9							y		
Cxx_121							y		
Cxx_123							y		
Cxx279							y		
FH2145							y		
INU055							y		
REN169O18							y		
REN247M23							y		
REN54P11							y		
VWF			y				y		
FH2017			y						
FH2087			y						

NGC – National Genomics Center (US)
ISPRA – Istituto Superiore per la Protezione e la Ricerca Ambientale (IT)
Senckenberg – Senckenberg Research Institute (DE)

VUW – University of Veterinary Medicine Vienna (AT)
UNIL – Université de Lausanne (CH)
UL – University of Ljubljana (SI)
OFB-ANTAGENE – Office français pour la biodiversité and the ANTAGENE lab (FR)
RAVA lab – Regione Autonoma Valle D'Aosta (IT)