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Technical report

Cutting edge genetic tools for fast, reliable and cost-effective surveillance of wolf conservation status and detection of hybridization with domestic dogs

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List of abbreviations

BC1w	first-generation backcross to wolves (F1 x wolf cross)
BC2w	second-generation backcross to wolves (BC1w x wolf cross)
eDNA	environmental DNA
F1	first-generation wolf-dog hybrid (wolf x dog cross)
HTS	high-throughput sequencing
LECA	Laboratoire d'Ecologie Alpine (France)
NGS	next generation sequencing
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
STR	short tandem repeats (also referred to as microsatellites)
UL	University of Ljubljana (Slovenia)
UNIL	University of Lausanne (Switzerland)
WDH	wolf-dog hybrids

Summary of contents

1. Introduction and objectives of the document

Many terrestrial species have large ranges and can travel hundreds of kilometers (e.g., Ražen et al., 2016; Bartoń et al., 2019; Fuglei & Tarroux, 2019). The movement of long-distance dispersers can therefore present an important challenge for species monitoring programs that occur only within national boundaries (Konec et al., 2024). Moreover, noninvasive genetic monitoring based on genotyping of microsatellite markers using capillary electrophoresis has been hampered by labor-intensive data processing and the need to calibrate genotypes between laboratories (De Groot et al., 2016), which has also been a barrier to rapid exchange of data among countries. This is an additional concern for accurate identification of dispersing individuals whose genetic profiles diverge from those seen in the local population where these dispersers pass through or settle. For example, in cases where the relevant wolf (*Canis lupus*) reference populations have not been included in analyses of population genetic structure, immigrant wolves risk being misclassified as admixed individuals with dog (*C. l. familiaris*) ancestry (Harmoinen et al., 2021). Therefore, a genotyping method that facilitates accurate and timely identification of wolf-dog hybrids (WDH), a conservation concern, from that of wolf dispersal and gene flow among genetically diverse populations, which is widely considered a conservation benefit, would also reduce the risk of detrimental management decisions.

A new genotyping approach based on high-throughput sequencing (HTS) of microsatellites, recently developed for brown bears (*Ursus arctos*), a wide-ranging species with several transboundary populations in Europe, has overcome many of the limitations of the standard microsatellite genotyping method using capillary electrophoresis (De Barba et al., 2017). The main feature of this new approach is to provide access to the sequence of the alleles, rendering the genotype data analyzed in different laboratories directly comparable and exchangeable without the need for calibration. In addition, because sequence data can be treated bioinformatically, the genotyping process can be automated and becomes very cost-effective even for very large sample sizes. Importantly, the new HTS approach may offer particular benefits to the study of wolves. An earlier European-wide assessment (Hindrikson et al., 2017) noted that approaches toward meta-analyses, such as the 'yardstick' method presented by Skrbinšek et al., (2012), can be limited by the larger number of available genetic markers for wolves than for bears, resulting in fewer overlapping markers among wolf studies. This method has now been applied to both bears (Skrbinšek et al., 2012) and wolves (Jan et al., 2023) across Europe. Whereas the analysis of bears permitted comparison of at least n = 6 microsatellite loci (Skrbinšek et al., 2012), the analysis of wolves integrated studies that shared only n = 4 microsatellite loci (Jan et al., 2023), highlighting the need for more harmonization (De Groot et al., 2016).

An additional benefit of having access to the actual sequence of individual alleles through the HTS method is the discovery of genetic diversity and evolutionary history that remain 'hidden' in standard capillary electrophoresis studies. With the latter, alleles are categorized solely by fragment length polymorphisms, whereas the HTS method also incorporates the actual sequence polymorphism and structure. Homoplasy, the occurrence of identical alleles with different evolutionary histories, is a

general limitation for microsatellites, which have high mutation rates (Putman and Carbone, 2014). However, the HTS genotyping method offers a more powerful approach for detecting genetic diversity and incorporating evolutionary history into population genetic analyses also from non-invasive sampling.

In the Alpine region, wolves from long-isolated populations have reconnected (Ražen et al., 2016). Rapid recolonization has resulted in the Alpine population spreading over multiple countries and national jurisdictions, where efficient monitoring and population size estimates are important and have set an example for other areas (Marucco et al., 2023). Transparent and reliable scientific data can also help reduce speculation and human-wildlife conflict associated with wolves dispersing into areas where they have long been absent or rare (Ciucci et al., 2009). In such areas, the ability to harmonize data collection and quickly assess the genetic profiles of putative dispersers against that of local reference populations is essential for timely and clear public communication.

The aim of the LIFE WolfAlps EU project is to promote human and wolf coexistence in the Alpine region. To facilitate transboundary population monitoring at a biologically meaningful scale, an important aim of this project has been the development of optimal HTS markers for wolves, especially for use with non-invasive samples (De Barba et al., 2024; De Barba et al., *in prep*). The objectives of this report are therefore to present (1) the methodological approaches used for wolf HTS marker development, (2) the work undertaken to test the HTS makers and optimize them to permit continued cost-effective monitoring after the LIFE WolfAlps EU project, and (3) provide examples of the successful use of these markers in practical population monitoring, identification of wolves from various population genetic clusters in the wider region, and identification of individuals with wolf-dog ancestry.

Photo: Francesco Panuello

2. Development of the method

Microsatellite (short tandem repeats - STR) markers were already developed for optimal genotyping based on high-throughput sequencing (HTS) in brown bears (De Barba et al., 2017) and the method was successfully put in practice in a large monitoring project (Skrbinšek et al., 2017). The aim here was to develop the same method also for wolves. New STR markers were developed and tested for optimal genotyping based on HTS, and laboratory protocols for the new genotyping method were set up and optimized. Bioinformatic pipelines for STR genotyping from HTS data were optimized to increase genotyping accuracy and computational efficiency. For this task, the University of Ljubljana (UL) cooperated closely with the Laboratoire d'Ecologie Alpine (LECA) and the University of Lausanne (UNIL).

2.1 Marker development

A wolf tissue sample from Switzerland was used for preparing a shotgun library for microsatellite (Short Tandem Repeats - STRs) identification and sequenced on an Illumina sequencing platform. We used a bioinformatic pipeline on the resulting sequence data, to design 200 STR markers (perfect tetranucleotides, maximum length 14-16 repeats) that were tested for co-amplification in 4 multiplex PCRs of 50 loci each, using a mixture of 5 wolf scat samples collected in Switzerland. The amplification products were sequenced in a single library on an Illumina platform. The sequence output was treated bioinformatically. Based on amplification performance (number of reads) and the ratio between authentic allele vs. artifact reads, we selected 50 loci out of the 200, which were amplified in the same 5 scat samples separately and sequenced in five distinct libraries on an Illumina platform. The results from this step were used to adjust primer concentrations and discard markers with high levels of artifacts. The remaining markers were amplified in two new scat samples and one sample from the previous tests, in 4 replicates each, and were sequenced in 9 libraries. A final set of 44 STR markers that can be co-amplified in a single multiplex PCR reaction was selected for HTS wolf genotyping. For efficient routine processing of large sample sizes, the primers of these 44 selected markers were modified by the addition of molecular identifier tags allowing sample multiplexing during sequencing. A sex marker for sex determination was also designed to be co-amplified and sequenced with the STR loci.

2.2 Laboratory protocol optimization

We optimized the laboratory protocols to improve co-amplification performance of a high number of loci with noninvasive (low quality/quantity DNA) genetic samples for efficient processing. This involved various trials of adjustments of the PCR protocol employed (amplification kit and specific reagents used, primer concentrations, thermocycler profile). The other front of optimization concerned the PCR-free library preparation step for sequencing to avoid tag jumps (low-level detection of alleles from different samples because of false DNA tag combinations that appear as a

biochemical artefact). The PCR-free library preparation protocol based on a combination of a recently published protocol (TagSteady, Carøe & Bohmann, 2020) and the Illumina protocol was developed at the University of Lausanne facility (De Barba et al., 2024).

2.3 Bioinformatics

In close cooperation with LECA and UNIL we optimized a bioinformatic pipeline for automated marker development from shotgun sequence data (De Barba et al., *in prep.*). This pipeline was used to design new markers for HTS genotyping as described above. We also considerably improved the existing genotyping pipeline, particularly to make it more tailored to the HTS data and increase genotyping accuracy and computational efficiency (De Barba et al., 2024). The bioinformatic procedure is described in Annex 2.

3. Final protocol

The usual and most optimal working unit is the 96-well PCR plate: 96 samples (including biological samples and controls) are processed at one time. We are using the multitube approach (Taberlet et al., 1996), typically performing 8 replicates per sample. Eight PCR plates are sequenced together in the same sequencing library on an Illumina platform. Tagged primers are used in the PCR to uniquely label each PCR product in the sequencing library and allow assignment of sequences to samples and markers in post-sequencing bioinformatic analysis. The protocol was presented at a genetic workshop organized by UL and a detailed protocol is given in Annex 1.

Figure 1: Laboratory preparation of tagged primers and PCR at the University of Ljubljana (UL). Photo: Marjeta Konec

3.1 New wolf STR and sex markers

The wolf STR (microsatellite) panel for HTS is comprised of 44 newly developed STR markers amplified in 2 multiplex PCR (22 loci per multiplex PCR), named multiplex A and B (see Table 1). The multiplex A of 22 STR loci is co-amplified with the sex marker and is effective for routine analysis of individual identification as well as for assessment of population genetic status, genetic structuring and pedigree reconstructions. Multiplex B consists of "redundancy" STR markers, which can be combined with markers in Multiplex A if more genetic data is needed to answer specific questions.

Table 1: List of the new STR (microsatellite) panel for high throughput sequencing, markers are divided into two multiplexes. For routine analyses, the multiplex A of 22 loci + sex marker is sufficient for individual identification and sex determination.

Multiplex A		**Multiplex B**	
Locus name	**STR motif**	**Locus name**	**STR motif**
Cl147	GATA	Cl109	TAAA
Cl233	AAAG	Cl113	ATAA
Cl264	ATAG	Cl178	AAAG
Cl274	AAGA	Cl211	GAAA
Cl285	AAAG	Cl218	AGAC
Cl291	AAAG	Cl226	GATA
Cl308	AAAG	Cl228	GAAA
Cl330	AAAG	Cl234	AAAG
Cl344	AATA	Cl251	AATA
Cl366	GAAA	Cl259	GGAA
Cl370	AAGA	Cl290	AAAT
Cl375	TAGA	Cl318	AAAT
Cl380	TAAA	Cl322	AAAT
Cl408	AAGA	Cl324	GAAA
Cl507	GAAA	Cl345	GAAA
Cl527	GAAA	Cl406	GAAA
Lup01	AGAA	Cl423	AAAT
Lup02	GTTT	Cl434	TAAA
Lup13	AATC	Cl441	GAAA
Lup20	TCAT	Cl517	ATAA
Lup21	ATGA	Cl523	GGAA
Lup23	GGAT	Lup15	CATC
ZFX_NEW	Sex ID		
ZFY_NEW	Sex ID		

Tagged primers, modified by the addition of molecular identifiers on the 5' end, are used in each PCR to uniquely label any given PCR product for retrieving the respective sequence data in post-sequencing bioinformatic analysis. Tags consist of eight nucleotides enabling a minimum of five mismatches between any pair of tags (Coissac, 2012). An additional 1–2 specified nucleotides were added to the tags 5' end to increase complexity for cluster detection on the flow cell.

Forward and reverse primers with the same tags are pooled into Primer Mixes (2x concentrated) and these are used to make Primer Plates (1x concentrated) used for PCR set up, where each individual PCR reaction (well on the PCR plate) has unique combinations of F and R tagged primers. With 32 F tags and 24 R tags, it is possible to make eight Primer Plates, corresponding to 32 x 24 = 768 unique tag combinations (Figure 2).

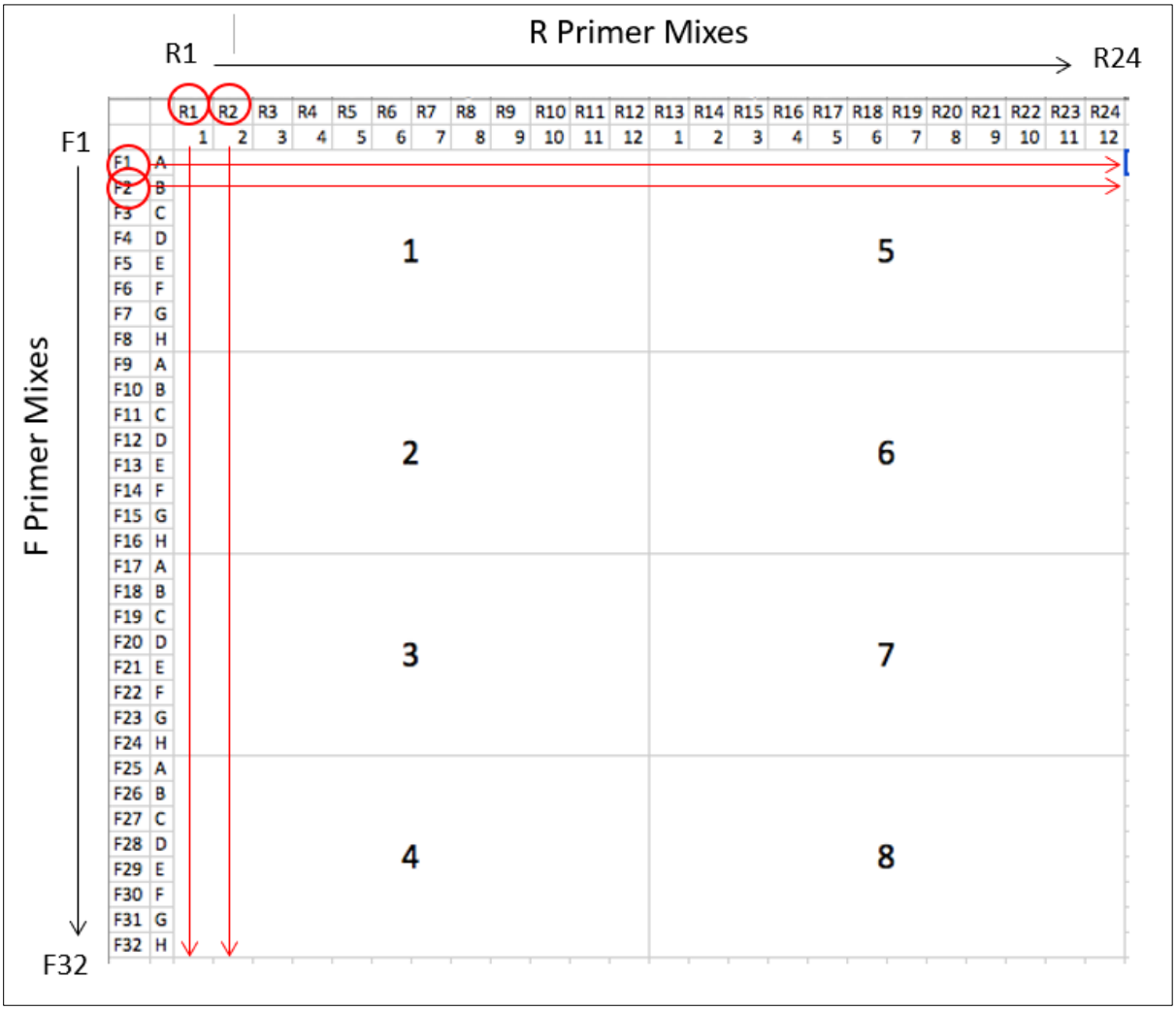


Figure 2: Preparation of 8 primers plates with 32 forward primer tags and 24 reverse primer tags.

3.2 DNA Aliquot Plates

DNA Aliquot Plates are 96-well plates containing aliquots of the DNA samples that will be used for the PCR. The volume depends on the amount of DNA template used in PCR and number of replicate PCR performed.

Besides the biological samples each aliquot plate must also include extraction negatives, two aliquots of a PCR negative control, two aliquots of a PCR positive control. We recommend using a positive control sample with a medium DNA quality index, resembling an average non-invasive sample, to balance the number of resulting reads. The plate also includes tagging system controls or "blanks", which are basically empty PCR wells, to monitor for tag jumps. Typically, 8 "blanks" are left diagonally in the plate. The Aliquot plate design is presented in Figure 3.

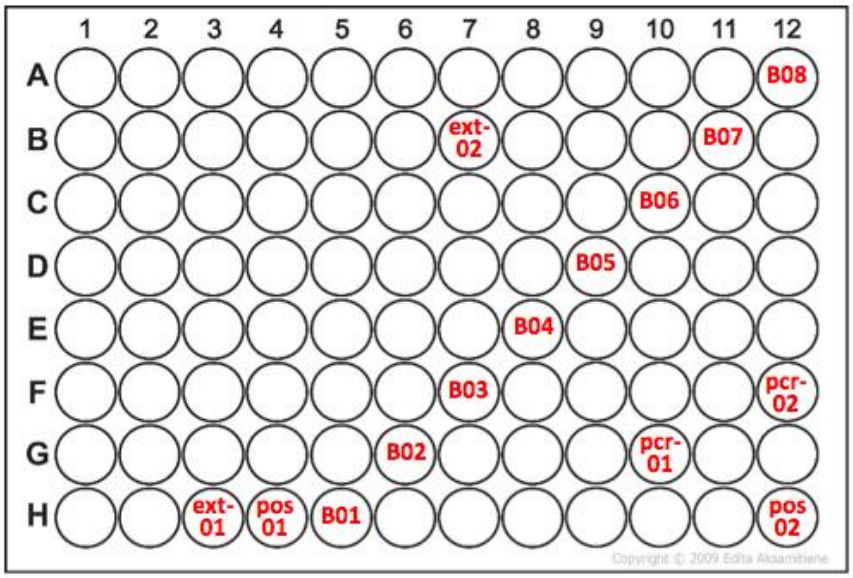


Figure 3: DNA aliquot plate design. B = tagging system controls (blanks), ext = extraction controls, pcr = PCR controls, pos = positive controls.

3.3 PCR

PCRs are carried out in a 10 µL volume and contain 1X concentrated Platinum Multiplex PCR Master Mix, 0.0032 mg BSA, 0.04-0.09 µM of each primer and 2 µL DNA template (diluted 1:1). The thermocycling profile has an initial denaturation step of 2 min at 95 °C, followed by 45 cycles of 30s at 95°C, 30s at 55°C, 60s at 72 °C and a final elongation step of 5 min at 72 °C.

PCR products from all eight primer plates are pooled together in a single library and purified using the MinElute PCR purification kit (QIAGEN GmbH). Concentration is measured with Qubit fluorometer (Life Technologies). To avoid tag jumps, the sequencing library for Illumina instrument should be prepared using a PCR-free procedure like TagSteady protocol (Carøe & Bohmann, 2020) enabling a significant reduction in the errors that otherwise occur during library preparation and sequencing (De Barba et

al., 2017). Currently two commercial services (iBioScience, Fasteris) offer this service. If the PCR-free library preparation is not available, much of the issue can be fixed by collecting more reads and through careful genotyping. More details of the protocol are given in Annex 1 and Annex 3.

3.4 Bioinformatic pipeline and allele calling

The results from the HTS sequencing on an Illumina platform are raw DNA sequences that need to be bioinformatically processed and transformed to consensus genotypes. The bioinformatic pipeline was presented in detail at the genetic workshop organized by UL and is described in Annex 2.

DNA sequence data analysis is performed using a modified version of the pipeline published in De Barba et al. (2017), implemented using in-house Python and R scripts, on a standard desktop computer running MacOSX.

Pipeline description and code is available at https://github.com/PazhenkovaEA/ngs_pipelines.py) (De Barba et al., 2024).

Initially, Illumina reads are processed using the OBITools3 (Boyer et al., 2016) to assemble paired-end reads, filter out unaligned sequences, demultiplex sequences by markers and samples discarding sequences without a perfect tag match and at least three primer mismatches. Demultiplexing of the sequences is schematically presented in Figure 4.

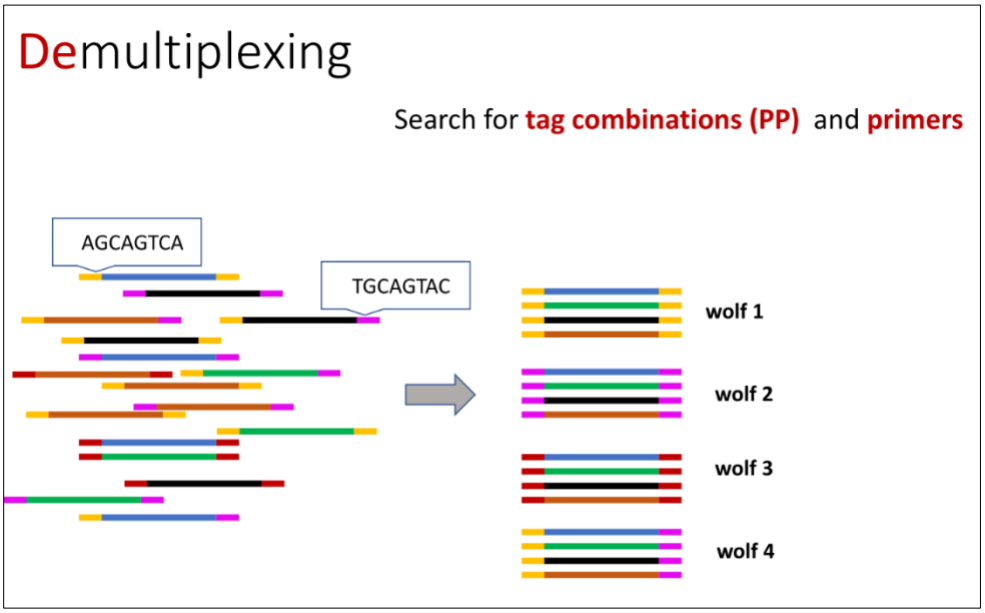
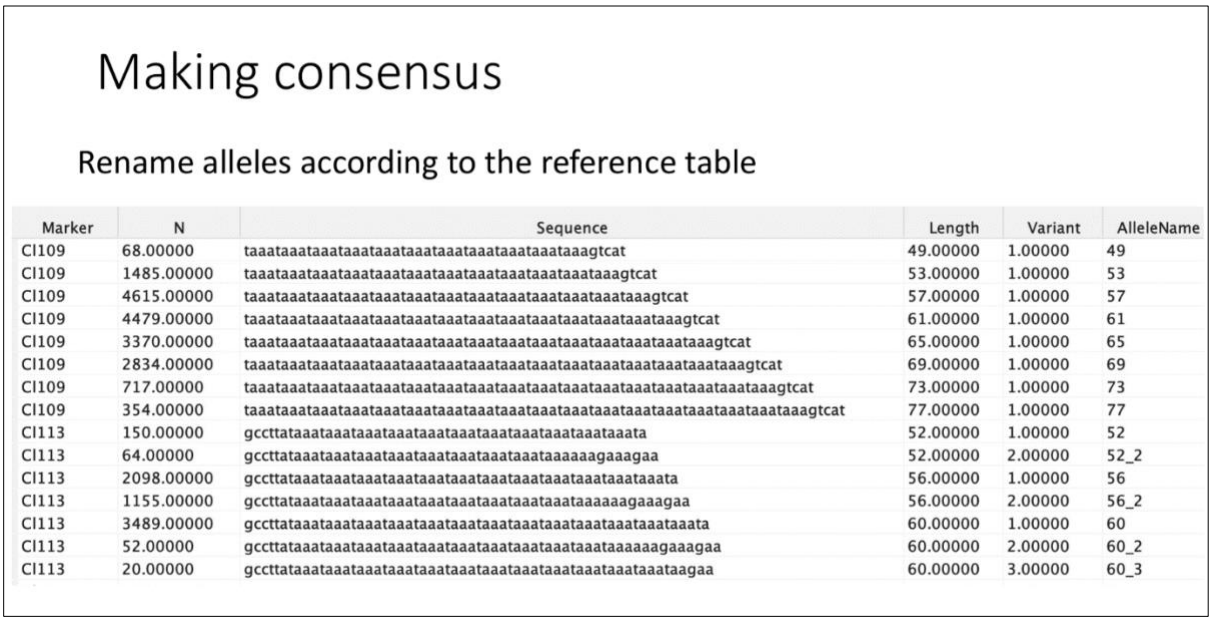


Figure 4: Demultiplexing the sequences.

STR alleles are inferred from the observed sequences and relative read counts in each PCR product following the process described in De Barba et al. (2017) and additionally optimized in this project (De Barba et al., 2024). In summary, alleles were defined as the most abundant sequences containing the

STR motif of the locus and associated to their relative stutter sequence. In the automated allele calling, the DNA sequence including the STR motif is given an allele name (Figure 5). If a sequence had no stutter and a lower number of reads than the user-defined threshold (default 100 reads), it was discarded. The threshold is adjusted to the noise caused by the tag-jumps that can be greatly reduced using the PCR-free library preparation protocol.



Making consensus

Rename alleles according to the reference table

Marker	N	Sequence	Length	Variant	AlleleName
CI109	68.00000	taaataaataaataaataaataaataaataaataaataaagtcat	49.00000	1.00000	49
CI109	1485.00000	taaataaataaataaataaataaataaataaataaataaataaagtcat	53.00000	1.00000	53
CI109	4615.00000	taaataaataaataaataaataaataaataaataaataaataaataaagtcat	57.00000	1.00000	57
CI109	4479.00000	taaataaataaataaataaataaataaataaataaataaataaataaataaagtcat	61.00000	1.00000	61
CI109	3370.00000	taaataaataaataaataaataaataaataaataaataaataaataaataaataaagtcat	65.00000	1.00000	65
CI109	2834.00000	taaataaataaataaataaataaataaataaataaataaataaataaataaataaataaagtcat	69.00000	1.00000	69
CI109	717.00000	taaataaataaataaataaataaataaataaataaataaataaataaataaataaataaataaagtcat	73.00000	1.00000	73
CI109	354.00000	taaataaataaataaataaataaataaataaataaataaataaataaataaataaataaataaataaagtcat	77.00000	1.00000	77
CI113	150.00000	gccttataaataaataaataaataaataaataaataaataaataaata	52.00000	1.00000	52
CI113	64.00000	gccttataaataaataaataaataaataaataaataaataaaaaagaaagaa	52.00000	2.00000	52_2
CI113	2098.00000	gccttataaataaataaataaataaataaataaataaataaataaataaata	56.00000	1.00000	56
CI113	1155.00000	gccttataaataaataaataaataaataaataaataaataaaaaagaaagaa	56.00000	2.00000	56_2
CI113	3489.00000	gccttataaataaataaataaataaataaataaataaataaataaataaata	60.00000	1.00000	60
CI113	52.00000	gccttataaataaataaataaataaataaataaataaataaataaaaaagaaagaa	60.00000	2.00000	60_2
CI113	20.00000	gccttataaataaataaataaataaataaataaataaataaataaataagaa	60.00000	3.00000	60_3

Figure 5: Naming the alleles from the raw sequences.

Consensus genotypes at each locus for a sample are determined based on STR sequence alleles observed across the eight PCR replicates, requiring that an allele be observed at least twice for heterozygotes and three times for homozygotes. Similarly, with the sex marker, males were scored by the detection of the homologous X and Y sexual chromosomes sequences in at least two replicate PCRs, while females are scored by the detection of the X chromosome sequence in at least three replicate PCRs. After the production of consensus genotypes, we visually checked the genotypes with plots (see Figure 6) and, where necessary, corrected the final genotype. Samples with quality index (QI) (Miquel et al., 2006) below 0,10 were discarded.

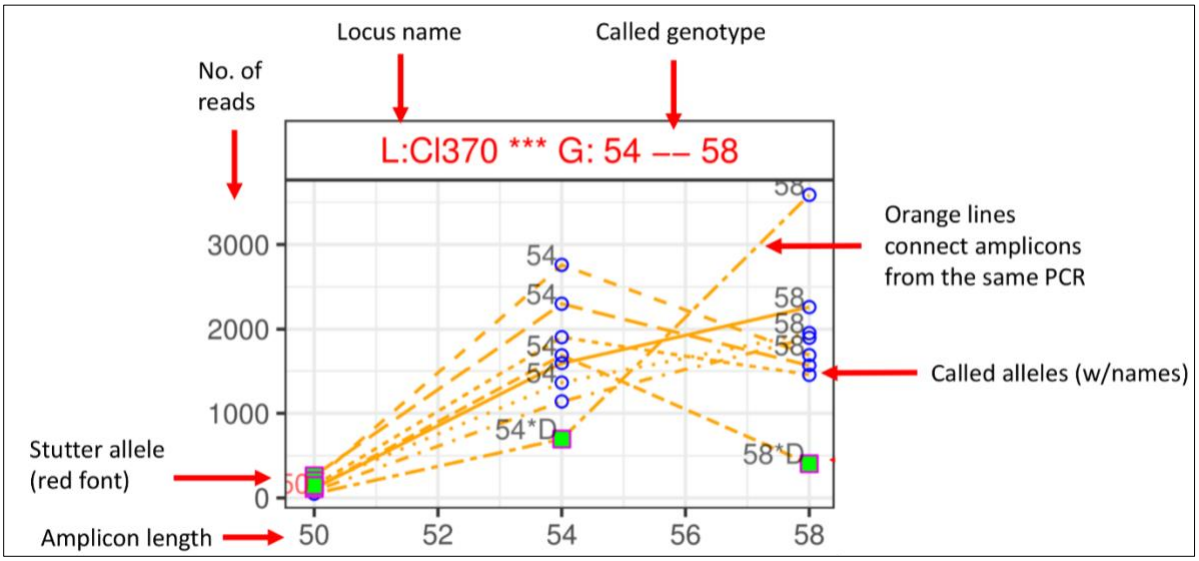


Figure 6: Genotype plots allow rapid visual assessment of the genotyping results.

Individual identification, matching of sample genotypes, calculation of genotyping success and error rates were done within a custom Microsoft Access database (Skrbinšek, unpublished) accounting for sample genotype reliability, locus mismatches, and probability of identity (Waits et al., 2001; Miller et al., 2002; Paetkau, 2003). Individual animals were assigned a unique code that was associated with all samples detected for that individual (AnimalRef).

4. Method implementation

4.1 Samples, reference populations

The first test of the method was applied to wolf samples (n=88) collected in the Alpine area of Slovenia in 2022, where 70 out of 88 samples were successfully genotyped. For this we only applied the multiplex A panel of markers and the sex ID marker.

In the next step we genotyped animal reference samples analyzed at UL from previous sampling seasons in Slovenia and Croatia to ensure data continuity and permit analyses that include samples collected before and after the implementation of HTS in regional monitoring. In this step, we also included the multiplex B of STR markers. Therefore, all genotyping data generated at UL are now backwards compatible and can be used in matching of the samples and for pedigree reconstructions, and altogether more than 500 samples were analyzed in this step.

To obtain references for other wolf populations, with the aim of reliable detection of (i) WDH and (ii) wolf migrants from other populations, reference samples from other populations in Europe were genotyped with the new method. This includes Alpine (samples from Italy), Central European (samples from Germany) and the Dinaric-Balkan region (samples from Slovenia, Croatia and Serbia), where previous research has shown there are three distinct genetic clusters present (Šnjegota et al., 2021). Additionally, outside of the LIFE WolfAlps EU project, this method has been replicated by genotyping of samples from the Carpathian population in Slovakia and Romania (unpublished data).

4.2 Species discrimination

Because STR markers developed in one canine species are known to amplify also in other canids (Stronen et al., 2020), we also genotyped reference fox (*Vulpes vulpes*) tissue samples (n=12) and golden jackal (*C. aureus*) tissue samples (n=19), which are the most frequent non-target species sampled in regional genetic monitoring of wolves. The golden jackal samples were selected to include individuals from both detected population clusters in our sampling area (the Dalmatian and Pannonian populations; Stronen et al., 2021).

The amplification success of the tested 44 STR markers in fox samples was on average 50.5%. However, STR markers that work well are highly polymorphic and several loci have private alleles specific for foxes. Such loci are therefore very informative for species identification, also for poor-quality samples where only incomplete genotypes have been obtained (e.g., saliva samples from livestock damage cases, UL, unpublished data).

In the golden jackal, the amplification success of the tested 44 STR markers was on average 74%, with several loci showing jackal-specific alleles.

A principal coordinate analysis clearly distinguishes fox and jackal samples from wolves and dogs (Figure 7), so the non-target species collected in the field can be reliably identified and excluded from further analyses.

Additionally, the ZF sex marker has a specific nucleotide sequence in both X and Y chromosomes in foxes, making it very useful for identification of this species. The nucleotide sequence is also specific in the Y chromosome of jackals, although there is no difference in ZF nucleotide sequences in wolves and dogs.

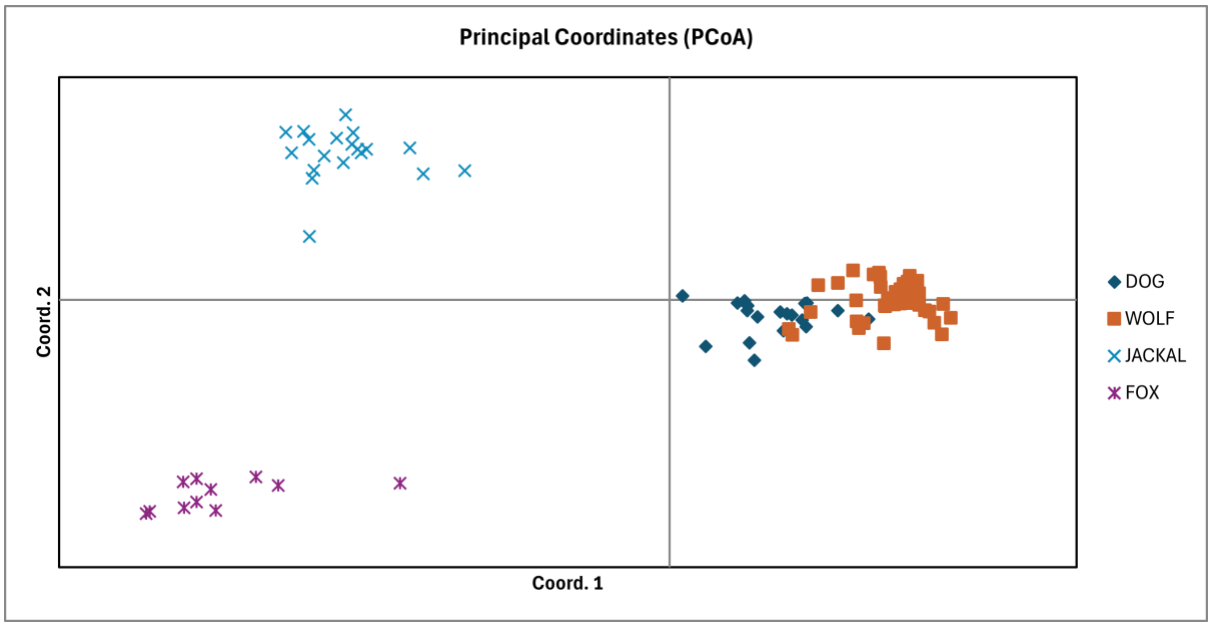


Figure 7: A principal coordinate analysis (PCoA) clearly distinguishes fox and jackal samples from wolves and dogs.

4.3 Genotyping success and use in a practical monitoring program

The new HTS genotyping method was successfully implemented for wolf monitoring in Slovenia in the 2022/2023 season (Annex 4), where more than 600 genetic samples were included in the study (Bartol et al., 2023). Considering only the target species (wolf and WDH) the effective success rate was 74.4%. This is considerably higher than the effective success rates in previous years (49.1% - 66%), when the capillary electrophoresis markers were used (Jan et al., 2023).

Because the STR markers amplify also in other canid species, non-target species collected in the study area can be identified by genotyping. Alongside the wolves, percentages of other species collected were 7.8% for foxes, 2.3% for dogs, and 0.35% for jackals. Accounting also for so-called mixed samples (where DNA of two separate individuals is present), the total genotyping success rate in the study is

85%. This is significantly higher than the success rate in previous sampling seasons (64.5% - 79.1% total success rate) and, in fact, the highest ever achieved for wolf monitoring in Slovenia.

Additionally, the method has been applied in Croatian wolf monitoring for samples collected in 2022/2023, where the total success rate was over 85% (Skrbinšek et al., 2023).

4.4 eDNA

Environmental DNA (eDNA) methods have been advancing rapidly in recent years, opening new opportunities in non-invasive studies of wildlife. We successfully applied the new STR markers in eDNA analysis of snow tracks. We have presented successful individual genotyping of eDNA obtained from snow tracks of three large carnivores: brown bears, Eurasian lynx (*Lynx lynx*) and wolves in a recent scientific paper (De Barba et al., 2024). The genotyping success rate in wolf samples was 70%, showing the usefulness of the method in future research, thereby advancing eDNA-based individual and population- level studies. The full peer reviewed paper is available in Annex 3.

Figure 8: Sampling snow tracks in the field. Photo: Jaka Črtalič

4.5 Characteristics of the new markers

Most of the newly developed markers were considerably polymorphic in all sampled populations. We thoroughly optimized and tested multiplex A (Table 2), which is sufficiently informative for routine wolf monitoring (individual identification, pedigree reconstruction, detection of hybrids). Locus Cl370 has amplification problems and is difficult to genotype, other loci are amplifying well and produce clear genotypes.

The additional markers in Multiplex B (Table 3) are less thoroughly optimized. They are designed to be used if additional genetic data is required and can be multiplexed with the loci in Multiplex A if needed. Out of the 22 markers in that multiplex, 14 are already suitable for practical use, 8 would require further optimization and 2 did not amplify (Cl423, Cl434) and are currently excluded from the report.

Detected alleles with their frequency and sequences are provided in Annex 5.

Table 2: Characteristics of the newly developed markers in different populations, Multiplex A (n = 22 markers). Din = Dinaric, CE = Central European, Alp = Alpine, PID = probability of identity, PIDsib = probability of identity for siblings. Total numbers of genotyped individuals: Dinaric = 772, Central European = 111, Alpine = 73.

	Expected Heterozygosity			Observed Heterozygosity			Allelic Diversity			PID			PID Siblings		
Marker	**Din**	**CE**	**Alp**	**Din**	**CE**	**Alp**	**Din**	**CE**	**Alp**	**Din**	**CE**	**Alp**	**Din**	**CE**	**Alp**
Cl147	0.80	0.85	0.74	0.78	0.80	0.74	19	16	11	0.07	0.04	0.09	0.37	0.33	0.40
Cl233	0.69	0.80	0.67	0.68	0.76	0.63	9	13	8	0.13	0.07	0.15	0.44	0.37	0.45
Cl264	0.85	0.74	0.81	0.82	0.73	0.78	20	15	12	0.04	0.10	0.06	0.34	0.40	0.36
Cl274	0.63	0.64	0.60	0.60	0.55	0.64	7	6	4	0.21	0.19	0.25	0.49	0.47	0.51
Cl285	0.78	0.78	0.75	0.75	0.72	0.73	22	12	6	0.08	0.08	0.10	0.38	0.38	0.40
Cl291	0.79	0.78	0.78	0.76	0.65	0.63	15	10	9	0.07	0.08	0.08	0.37	0.38	0.38
Cl308	0.70	0.78	0.54	0.68	0.74	0.58	9	12	4	0.13	0.08	0.31	0.44	0.38	0.55
Cl330	0.85	0.86	0.72	0.71	0.78	0.62	19	14	13	0.04	0.03	0.11	0.34	0.33	0.42
Cl344	0.69	0.81	0.70	0.66	0.82	0.59	8	13	6	0.14	0.06	0.14	0.44	0.36	0.44
Cl366	0.71	0.66	0.51	0.71	0.66	0.49	5	6	3	0.14	0.18	0.36	0.43	0.46	0.58
Cl370*	0.43	0.65	0.48	0.10	0.30	0.17	13	9	4	0.34	0.18	0.34	0.62	0.47	0.59
Cl375	0.78	0.78	0.60	0.78	0.74	0.59	15	11	7	0.08	0.08	0.22	0.38	0.38	0.50
Cl380	0.76	0.81	0.63	0.66	0.72	0.53	15	12	4	0.08	0.06	0.22	0.39	0.36	0.49
Cl408	0.82	0.83	0.54	0.80	0.77	0.53	9	11	4	0.06	0.05	0.28	0.35	0.35	0.55
Cl507	0.77	0.85	0.60	0.75	0.68	0.58	14	15	8	0.09	0.04	0.19	0.39	0.33	0.50
Cl527	0.84	0.78	0.67	0.72	0.65	0.63	19	18	7	0.04	0.07	0.16	0.34	0.38	0.45
Lup01	0.75	0.59	0.52	0.19	0.34	0.40	10	9	7	0.10	0.23	0.31	0.40	0.52	0.57
Lup02	0.51	0.70	0.62	0.45	0.59	0.58	5	6	5	0.29	0.14	0.21	0.57	0.44	0.49
Lup13	0.50	0.66	0.55	0.50	0.65	0.56	4	4	3	0.30	0.19	0.28	0.57	0.47	0.54
Lup20	0.68	0.56	0.60	0.66	0.58	0.57	7	5	4	0.15	0.27	0.23	0.45	0.54	0.51
Lup21	0.75	0.72	0.71	0.73	0.74	0.65	7	9	6	0.09	0.11	0.12	0.40	0.42	0.42
Lup23	0.65	0.61	0.51	0.57	0.29	0.29	5	5	3	0.20	0.24	0.36	0.48	0.51	0.59

** Marker is difficult to genotype, possible null alleles.*

Table 3: Characteristics of the newly developed markers in different populations, Multiplex B (n = 20 markers). Only individuals from the Dinaric population were genotyped with this multiplex. He = expected heterozygosity, Ho = observed heterozygosity, A = allelic diversity, PID = probability of identity, PIDsib = probability of identity for siblings. N=114 individual animals were genotyped.

Locus	He	Ho	A	PID	PIDsib
Cl109*	0.76	0.43	5.00	0.10	0.40
Cl113*	0.78	0.39	7.00	0.08	0.38
Cl178	0.84	0.26	15.00	0.04	0.34
Cl211	0.71	0.39	6.00	0.13	0.43
Cl218	0.82	0.12	12.00	0.05	0.35
Cl226	0.54	0.45	8.00	0.25	0.54
Cl228	0.67	0.43	6.00	0.17	0.46
Cl234	0.54	0.39	9.00	0.30	0.55
Cl251*	0.00	0.00	1.00	1.00	1.00
Cl259	0.68	0.61	6.00	0.16	0.45
Cl290	0.67	0.00	3.00	0.19	0.46
Cl318*	0.71	0.38	4.00	0.13	0.43
Cl322*	0.00	0.00	1.00	1.00	1.00
Cl324	0.80	0.34	9.00	0.07	0.37
Cl345	0.83	0.78	10.00	0.05	0.35
Cl406	0.78	0.80	12.00	0.07	0.38
Cl441	0.82	0.74	18.00	0.05	0.36
Cl517*	0.64	0.47	4.00	0.18	0.48
Cl523	0.69	0.62	7.00	0.14	0.44
Lup15	0.71	0.68	5.00	0.14	0.43

* Locus is not amplifying well and requires further optimization

4.6 Detection of genetic structuring with the new marker panel and detection of wolf-dog hybridization

We tested performance of Multiplex A for detecting genetic structuring in wolves, which includes detection of WDH. Detection of genetic structure is important to understand the development of fragmented wolf populations and detect gene flow between populations and population fragments. An understanding of genetic structure is also important in detection of WDH, where highly valuable immigrants from other wolf populations can be misclassified as WDH.

The marker panel clearly recognized structuring in the study area encompassing the Alpine, Central European and Dinaric-Balkan regions, including the substructure within the Dinaric-Balkan region (Figures 9 and 10). It also detected animals of mixed Alpine-Dinaric ancestry which are now colonizing the eastern Italian Alps and appearing in western Slovenia. Interesting is also the detection of several animals that seem to have some Central European ancestry in the Dinaric-Balkan region, particularly in samples collected in Serbia, including one animal that seems like a direct 1st generation descendant.

However, this result should also be checked in further analyses including additional European populations. Nevertheless, this might indicate some geneflow from the Central European wolves into the Dinaric-Balkan region, which to our knowledge has not been described yet but would not be surprising considering the large distances crossed by dispersing wolves (Ražen et al., 2016; Konec et al., 2024).

For detection of wolf-dog hybridization, the marker system reliably detected the first-generation (F1) hybrids and the first-generation backcrosses to wolves (BC1w) from a known hybridization event in Slovenia. For second-generation backcrosses (BC2w), parallel analyses with STR and the reduced panel of single nucleotide polymorphism (SNP) markers designed for WDH detection (Harmoinen et al., 2021) showed that the STR panel became unreliable. Here the STR panel misclassified some non-admixed animals as BC2w, and it also missed some real BC2w and classified them as non-admixed wolves. At the BC2w level of hybridization this marker system can possibly detect suspicious animals, but additional confirmation is recommended before any management action is taken into consideration. The usefulness of the marker system for detection of suspect BC2w individuals needs to be further evaluated, and such efforts are already underway.

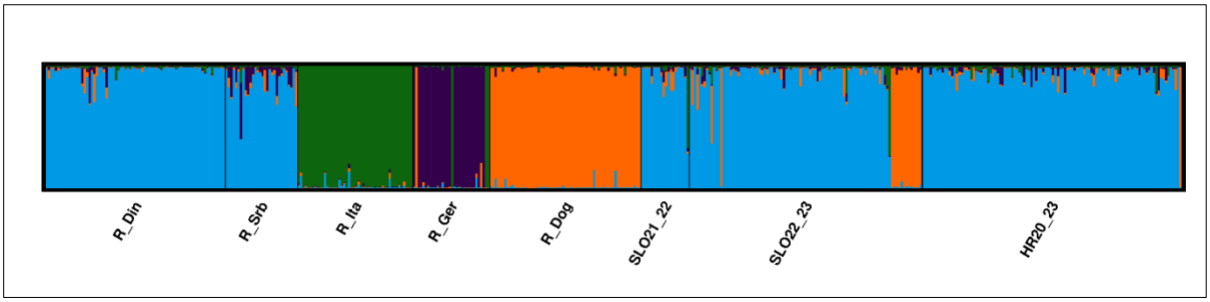


Figure 9: STRUCTURE plot, detection of genetic structure and wolf-dog hybridization, K=4. Each vertical line is an individual, the proportion of color indicates probability of assignment to a certain cluster. R_Din = reference Dinaric (Slovenia + Croatia), R_Srb = reference Serbian, R_Ita = reference Alpine, R_Ger = reference Central European, SLO21_22 = field samples, Slovenian wolf monitoring 2021/2022, SLO22_23 = Slovenian wolf monitoring 2022/2023, HR20_23 = Croatian wolf monitoring 2020-2023. R_Ger includes a jackal sample clustered with dogs (orange; jackal references were not included here, but see Figure 7), and 3 animals from the Alpine population. Field samples from Slovenia and Croatia include dogs and wolf-dog hybrids from a known hybridization event (F1, BC1w), and a few animals with suspect ancestry (BC2w).

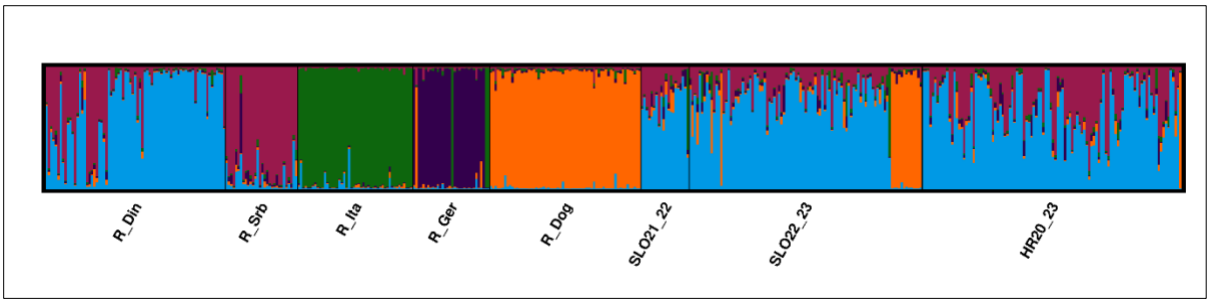


Figure 10: STRUCTURE plot, detection of genetic structure and wolf-dog hybridization, K=5. With the hypothesis of 5 clusters, the markers start to detect population substructure within the Dinaric-Balkan region (Šnjegota et al., 2021).

5. Conclusions

Standardization of genetic monitoring between different laboratories and across borders has long been an outstanding issue for transboundary wolf monitoring. This highly mobile species that is rapidly recolonizing the European continent knows no borders, and monitoring should be both organized at the population level and harmonized at the level of European continent to understand this process and adapt wolf conservation and management accordingly. However, technical issues of data compatibility and transferability between laboratories have hindered these efforts in the past, prohibiting the transboundary genetic monitoring of wolves to develop to its full potential.

Through this action, we developed and thoroughly tested a new genotyping method, which was developed in collaboration with UNIL and LECA. It completely solves the data transferability problem and generates reproducible genotypes that can be immediately shared between laboratories. This enables direct genetic tracking of animals across national borders and therefore permits transboundary, population- or metapopulation-level monitoring of wolves at biologically relevant scales. As the data is at the level of DNA sequences and as such completely future-proof and platform independent, they also lay the foundation for longitudinal studies of wolves as the monitoring data accumulates over the years.

Whereas data transferability is a key advantage of the new method, there are other important benefits. We have observed considerably higher genotyping success rates than what we were achieving in the past, which increases the efficiency of monitoring and decreases its costs. Another advantage is scalability. The laboratory part of the method can be made considerably more efficient through laboratory automation, and data analysis, which previously required a lot of work by trained personnel, is now mostly automated through bioinformatics, where just the final checking of the genotypes requires a human operator. This allows rapid, cost-effective genotyping of large quantities of samples, opening new horizons for intensive monitoring of this species.

After the development of the method, we started applying it for real-world monitoring of wolves. In Slovenia, we fully switched to this method during the monitoring season 2022/2023. A similar switch has already been done by Switzerland and Croatia, and we are applying it also in smaller-scale projects in Romania (Transylvania) and Slovakia (High Tatra Mts.). Most laboratories dealing with analysis of wolf samples from the Alps took part in the training provided through the project, and there are initiatives underway beyond this LIFE project to standardize entire wolf genetic monitoring in the region around this method. We have reanalyzed with the new approach a subset of wolf samples already genotyped with the standard method by other laboratories of the Genetic Wolf Alpine Group. This will allow us to compare method performance to further inform its implementation by these labs, and to start re-genotyping legacy wolf samples in other parts of the Alps to ensure data continuity. Beside the Slovenian and Swiss laboratories that are routinely using the new method, the Natural History Museum in Vienna (Austria), the Edmund Mach Foundation in Trento (Italy), and Senckenberg Research Institute (Germany) have started implementing the method in their genetic laboratories. In

addition, these laboratories are coordinating the establishment of an international genetic database for wolves, which is currently under development with financial support by the ARGE ALP initiative.

Together with UNIL and LECA, we managed to develop an excellent tool for genetic monitoring of wolves that can finally bring transboundary genetic monitoring of this species to its full potential. Hence, we have laid the foundation for long-term, population-level monitoring programs, which will be one of the lasting legacies of this LIFE project.

6. List of annexes

Annex 1 – Wolf HTS microsatellite (STR) laboratory protocol

Annex 2 – Bioinformatic analysis of NGS data

Annex 3 – Peer reviewed scientific paper describing the method and use for eDNA analysis

Annex 4 – English summary of Slovenian wolf monitoring report for sampling season 2022/2023

Annex 5 – Detected alleles, their sequences and frequencies in different wolf populations

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