

Client: Province of Gelderland, Netherlands  
Contact: [REDACTED] Programma vitaal platteland, opdrachtgever natuurbeheer

Ljubljana, 12 Sep 2023

Offer No. 20230912

Valid until: 12 Oct 2023

**Offer for research into possible hybridization of wolves (*Canis lupus*) in the Netherlands**

As per inquiry, we're providing an offer to analyse 10 tissue samples of wolves from the Netherlands and to explore their hybridization status with genomic-level data.

Laboratory work

- DNA extraction from 10 samples
- Preparing & sending DNA extracts for genotyping to external provider

5.1.2f

Genotyping DNA extracts on Illumina CanineHD BeadChip, 170000 SNP markers

- Minimum batch size is 48 samples. The other positions will be filled with reference samples from other European relevant wolf populations, including known hybrid animals.

5.1.2f

Data analysis & reporting

- Data quality check & screening
- Analysis, principal component analysis (PCA), Bayesian clustering, comparison & population (hybridization) assignment with data from all relevant European wolf populations and reference dogs. Full genome-level dataset.
- Checking of assignments with the reduced panel of SNPs used for routine hybridization analyses.

5.1.2f

**Total**

**17526.00 EUR**

The offered price does not include the VAT. Time for completion of the task is 4 months from the delivery of samples to the laboratory.

We are looking forward to collaborating with you.

Sincerely,

[REDACTED]  
[REDACTED]  
Digital Signature  
Ljubljana, Slovenia  
2023-09-12 21:15:18  
[REDACTED]  
Director, DivjaLabs Ltd.

## Proposed methodology

We will extract genomic DNA from the provided 10 tissue samples. The provided samples will be genotyped using the Illumina CanineHD BeadChip, including over 170,000 single nucleotide polymorphism (SNP) markers, where existing data from several wolf populations across Europe<sup>1</sup> and a variety of dog breeds<sup>2</sup> are publicly available. As the minimum batch size for genotyping is 48 samples (technical limit), we will extend the available reference dataset by including samples of 38 individuals from other relevant populations and known wolf-dog hybrids, including back-crosses. After genotyping, the raw data will be screened using PLINK<sup>3</sup>, to retain only high-quality profiles with individual and per-SNP call rates of at least ninety percent.

Successful profiles will be analysed together with publicly available wolf and dog profiles to evaluate their ancestry, including species and population status, and potential hybrid origin. For these analyses we will use both (A) the genome-wide profiles that successfully pass the screening process described above and (B) markers in the panel of 96 SNPs previously found to be highly discriminant in detecting wild versus domestic canids<sup>4</sup>. Use of the smaller 96 SNP panel in (B) together with the genome-wide profiles will provide verification of the smaller panel for future routine use, as this smaller panel is more cost-effective and can be used with noninvasive genetic samples (saliva on killed prey, scat, urine in snow, hair).

Population analyses for both datasets will be done with complimentary tools, where we will first use principal component analyses (PCA) in the *adegenet*-package<sup>5</sup> in R<sup>6</sup>, which does not have any population genetic assumptions about equilibrium conditions and is very useful for detecting outlier profiles such as immigrant individuals. Next, we will for the genome-wide dataset in (A) use ADMIXTURE<sup>7</sup>, a maximum likelihood method developed for the detection of population structure in larger genomic data sets. For the 96-SNP panel we will use STRUCTURE<sup>8</sup>, a standard Bayesian method for detecting population structure that is well-suited for smaller data sets.

Based on these analyses we will assign canid genotypes to species and populations where relevant or note individuals that have profiles consistent with a first-generation hybrids (F1), a first-generation back-cross to wolves (i.e., offspring of a crossing between F1-individuals and wolves; BC1<sub>wolf</sub>) or dogs (BC1<sub>dog</sub>). The smaller panel (B) can typically be used to detect back-crosses until the 2<sup>nd</sup> generation back-cross to wolves (BC2<sub>wolf</sub>) of European origin<sup>4</sup>, but BC2<sub>wolf</sub> and BC3<sub>wolf</sub> back-crosses might be more

challenging to distinguish. In such cases the sample quality, and an individual's origin outside of the European populations used for the development of the panel (e.g. wolves originating from ZOO populations), could also play a role. The larger genome-wide dataset (A) will be used to assess population origin, and the extent to which profiles could indicate admixture among relevant European wolf populations, with focus on Northern European (Finland, Poland, Belarus, Russia), Carpathian, Dinaric, or Balkan wolves.

## References

- <sup>1</sup>Stronen, A. V. et al. (2013). North-south differentiation and a region of high diversity in European wolves. *PLoS ONE* 8(10), e76454.
- <sup>2</sup>Vaysse, A. et al. (2011). Identification of genomic regions associated with phenotypic variation between dog breeds using selection mapping. *PLoS Genet.* 7(10), e1002316.
- <sup>3</sup>PLINK 1.9, <https://www.cog-genomics.org/plink/>.
- <sup>4</sup>Harmoinen, J. et al. (2021). Reliable wolf-dog hybrid detection in Europe using a reduced SNP panel developed for non-invasively collected samples. *BMC Genomics* 22, 473.
- <sup>5</sup>Jombart, T. (2008). Adegnet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24, 1403–1405.
- <sup>6</sup>R Core Team (2020). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- <sup>7</sup>Alexander, D. H., Novembre, J. & Lange, K. (2009). Fast model-based estimation of ancestry in unrelated individuals. *Genome Res.* 19, 1655–1664.
- <sup>8</sup>Pritchard, J. K., Stephens, M. & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* 155, 945–959.