Guidelines for the standardisation of genetic analyses to detect wolf-dog hybrids across Europe

Wolfness Technical Report D2

Report prepared as part of the Biodiversa+ project Wolfness "*Preserving the natural heritage of wolves: a multidisciplinary approach towards effective and socially acceptable management of wolf-dog hybridization across Europe*" by

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GUIDELINES FOR THE STANDARDISATION OF GENETIC ANALYSES TO DETECT WOLF-DOG HYBRIDS ACROSS EUROPE

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Content

Executive summary

The Biodiversa+ Wolfness project is centred on investigating, raising awareness, and evaluating approaches to mitigating impacts of anthropogenic hybridisation in Europe through in-depth interdisciplinary research on hybridisation between wolves (*Canis lupus*) and domestic dogs (*C. l. familiaris* or C. *familiaris*). The first step in addressing wolf-dog hybridisation (WDH) is the ability to reliably distinguish hybrids and introgressed individuals from non-admixed wolves. Likewise, it is critical to promote standardisation of both laboratory and analytical procedures to ensure comparability of results among genetic laboratories and research groups, especially given the expanding and increasingly interconnected wolf populations across Europe. In October 2023, the Wolfness project organised a workshop in Gelnhausen, Germany, with the aim of (i) reviewing laboratory protocols and local experiences in WDH identification, (ii) standardising laboratory protocols and analytical procedures across Europe, and (iii) developing a shared definition of 'wolf-dog hybrid' for conservation and management.

1. Introduction

1.1. Background

Hybridisation—the interbreeding between individuals from genetically distinct lineages, regardless of their taxonomic status—with domestic or alien species presents an important threat to the long-term survival of wild species (e.g. Rhymer and Simberloff 1996, Allendorf et al. 2001, Todesco et al. 2016). Such human-induced or anthropogenic hybridisation occurs globally, across a wide array of taxa (Mallet 2005), and its extent is often underestimated (Allendorf et al. 2001).

Notably, researchers working with diverse species have reported that morphological or phenotypic information is not always reliable for the identification of hybrids and introgressed individuals (e.g. wild boar admixed with domestic pig, *Sus scrofa* and *S. scrofa domesticus*, Iacolina et al. 2018; wildcats admixed with domestic cats, *Felis silvestris* and *F. catus*, Senn et

al. 2019). Adopting morphological cues of hybridisation encompasses two types of misidentification: erroneously classifying individuals as admixed when they have no or negligible signatures of admixture (Type I error, also known as false positives) and, conversely, failing to detect admixed individuals (Type II error, also known as false negatives).

A major concern for wolf (*Canis lupus*) conservation in Europe is wolf-dog hybridisation (henceforth WDH). The lack of consistency in analytical protocols and detection criteria across different laboratories in Europe has limited progress in research and conservation actions (de Groot et al. 2016, Salvatori et al. 2020). Recent surveys have indicated that, at least in some areas, WDH is more prevalent than previously thought (Salvatori et al. 2019, Santostasi et al. 2021). Moreover, wolf recolonisation in Europe has resulted in range edges where the species now occur at low density and where WDH may occur more frequently, facilitated, among other factors, by a high availability of free-ranging dogs (Galaverni et al. 2017, Lobo et al. 2023).

The Biodiversa+ Wolfness project aims to address the conservation impacts of anthropogenic hybridisation in Europe by focusing on wolf hybridisation with domestic dogs (*C. l. familiaris* or *C. familiaris*; see Gentry et al. 2004). A first, fundamental step in addressing WDH is the ability to detect hybrids and introgressed individuals by reliably (i.e. with high statistical confidence) discriminating them from non-admixed wolves. In the light of the expanding and increasingly interconnected wolf populations across Europe, it is also crucial to promote standardisation of optimised laboratory and analytical procedures to ensure comparability of results among genetic laboratories and to avoid confounding hybridisation with natural gene flow from adjacent wolf populations.

In October 2023, the Wolfness project organised a workshop in Gelnhausen, Germany, hosted by the Senckenberg Center for Wildlife Genetics. The workshop objectives were to (i) review laboratory protocols and local experiences in WDH identification, (ii) standardise laboratory protocols and analytical procedures across Europe, and (iii) develop a shared definition of 'wolf-dog hybrid' for conservation and management (Stronen et al. 2025a,b).

A review of methods currently adopted for the detection of wolf-dog hybrids and introgressed individuals, and for the establishment of minimum Europe-wide assessment standards for

identification of wolf-dog hybrids, are addressed in Wolfness technical report D1 "Review of current methods for the genetic identification of wolf-dog hybrids in Europe and establishment of minimum assessment standards" (Birkenhain et al. 2025). The review provides essential background and context for these guidelines and subsequent Wolfness technical reports.

Additional considerations for sampling design, to ensure unbiased population-level estimates of admixture, are addressed in Wolfness technical report D3 "Sampling strategies to assess and monitor wolf-dog hybridisation at the population level" (Ciucci et al. *in prep*). WDH is a complex topic, and other aspects that are vital to examine, but outside the scope of these guidelines, include legal (Trouwborst et al. 2014, Fossati 2024), ethical (e.g. Dubois et al. 2017), and policy (Salvatori et al. 2020) considerations, which are addressed by other Wolfness work packages and actions.

1.2. Aims

The key aim of these guidelines, building on the Gelnhausen workshop objectives (i) and (ii) noted above, is to provide a framework for improved European standardisation of analytical protocols to detect wolf-dog hybrids. However, we acknowledge that, although standardisation of population genetic monitoring approaches has made considerable progress in some transboundary regions (De Barba et al. 2024), such broad-scale efforts across multiple jurisdictions and institutions are complex processes that require time (de Groot et al. 2016), and this is also the case for WDH detection.

Accordingly, we maintain the need for a pragmatic approach that, at least for the time being, continues to integrate the use of locally established genetic markers and analytical procedures. These can typically identify dogs, wolves, first-generation wolf-dog hybrids (F1), and backcrosses to wolves up to the second generation, as reported for various parts of Europe (e.g. Randi et al. 2014, Godinho et al. 2015, Caniglia et al. 2020). If the relevant wild and domestic reference populations are included in the analyses, it is also possible to distinguish immigrant wolves from other European populations[1] and their descendants. However, if the

[1] Immigrants will normally show divergent genetic profiles and may carry ancestry from multiple wolf populations (e.g. Konec et al. 2024).

relevant reference populations are not included and analysts instead choose to use other, more genetically divergent groups as references, the canid profiles being tested will have low statistical assignment probabilities to the chosen references. Such canids may be erroneously classified as individuals resulting from hybridisation among canid species, which in Europe might include wolves, domestic or stray dogs, and golden jackals (*C. aureus*, Galov et al. 2015, Gopalakrishnan et al. 2018, Pilot et al. 2018). Although our guidelines are focused on European wolves, the topic of wild-domestic hybridisation is also highly relevant beyond our study area (e.g. Kopaliani et al. 2014, Mallil et al. 2020, Cairns et al. 2021, Korablev et al. 2021) and focal taxonomic groups (e.g. Iacolina et al. 2019, Eliášová et al. 2022).

Whereas the 'wolf-dog hybrid' definition developed from the Gelnhausen workshop is presented in detail elsewhere (Stronen et al. 2025a,b), these guidelines build on the workshop summary document's first two objectives (Stronen et al. 2025a) to present the current status of standardisation and outline ongoing developments and future needs. The guidelines are intended for researchers, practitioners, managers, conservationists and others involved with, or interested in, questions concerning wolf-dog hybrids, with focus on European wolf populations. Building on the review provided in Wolfness technical report D1 (Birkenhain et al. 2025), we seek to make technical details about key genetic methods more available to non-geneticists, by including definitions and additional background information.

2. Glossary and key terminology

Hybrid: An individual with an intermediate genotype between two diverged, parental populations as a result of interbreeding between individuals from these genetically distinct populations (i.e. hybridisation).

Hybridisation: Crossbreeding of individuals from genetically diverged species and/or populations. When driven by human activities, this process is called anthropogenic hybridisation. Hybridisation occurs without further introgression of genetic material if first-generation hybrids are sterile.

Backcross: The offspring of a mating between a hybrid and an individual of one parental group. As an example, a “first generation backcross to wolf” (also referred to as BC1w) means the outcome of a mating between an F1 wolf-dog hybrid with a wolf, resulting in offspring with theoretically 75% ancestry from wolves and 25% from dogs (the probabilistic nature of inheritance means that these values are approximate). A “second generation backcross to wolf” (also referred to as BC2w) is the offspring of a BC1w and a wolf and has approximately 12.5% dog ancestry. Although, in a strict sense, the term "hybrid" only refers to offspring of the interbreeding between genetically distinct lineages, it is often used to refer to recent backcrosses. To facilitate communication with stakeholders, we propose to include the first two generations of backcrosses in the definition (see 3.1 and Stronen et al. 2025a,b).

F1: A first generation hybrid. Mating between a wolf and a dog will result in a F1 hybrid, and such individuals will have ancestry that are 50% dog and 50% wolf.

BC1w: A first generation backcross into the parental wolf population. That is, the result of the mating between an F1 individual and a wolf. BC1w individuals have approximately 25% dog ancestry.

BC2w: A second generation backcross into the parental wolf population. That is, the result of mating between a BC1w individual and a wolf. BC2w individuals have approximately 12.5% dog ancestry.

Introgression: The incorporation of genetic variants from genetically divergent taxonomic groups that is mediated by backcrossing of hybrids into the parental (wild) populations. Introgression of genetic material from one population or species into another may be a long-term consequence of hybridisation (i.e. introgressive hybridisation).

Hybrid swarm: An introgressed and hybridising population that includes F1 hybrids and various recent backcrosses owing to a total breakdown of assortative (i.e. non-random) mating. Also known as a unimodal hybrid zone.

Cryptic hybrid: An individual deemed a hybrid according to genetic criteria, but without obvious morphological cues of hybrid ancestry.

Type I error: The risk of erroneously rejecting the null hypothesis even though it is true. For the purpose of this text, a Type I error reflects the risk of erroneously identifying a wolf as a hybrid.

Type II error: The risk of erroneously accepting the null hypothesis even though it is false. For the purpose of this text, a Type II error reflects the risk of erroneously identifying a hybrid as a wolf (i.e. failing to detect a hybrid individual).

Reference population: Here defined as a set of wolves from the same genetically recognised population and that have no traces of dog admixture, that is used as reference genotype group in statistical admixture testing.

Alleles: The different variants of a gene.

Genotype: The genetic composition of an individual.

Phenotype: The set of traits an individual exhibits (e.g. morphology, physiology, behaviour), resulting from the individual's genotype and environmental factors.

Genetic markers: DNA sequences or genes at known chromosomal locations that can be used to identify individuals, populations or species. Typical examples of markers used in genetic monitoring are microsatellites (also referred to as short tandem repeats or STR) and single nucleotide polymorphisms (SNPs).

Microsatellites: Segments of DNA with repeating units of 1–6 base pairs, also known as short tandem repeats (STRs). The repeating nature of these DNA segments results in high mutation rates during DNA replication, which leads to variability within and between populations. This allows the identification of distinct individuals and populations using a limited number of these genetic markers.

SNP (single nucleotide polymorphisms): a genetic sequence variation affecting only one of the building blocks or bases (adenine (A), cytosine (C), guanine (G), and thymine (T)) in a DNA molecule.

Non-invasive genetic sampling: Where the source of the DNA is left behind and can be collected without disturbing or catching the individual. Common source materials for the

study of canid species are faecal materials (scats), hair, urine and saliva collected from prey at kill sites.

Assignment test: The use of individual genetic profiles and statistical programs to probabilistically assign them to the detected genetic clusters or populations (see Birkenhain et al. 2025, chapter 4.3 for additional information and examples).

3. Defining wolf-dog hybrids for conservation management

At the Gelnhausen workshop, discussions among wolf ecologists, geneticists and practitioners encompassing various European research, conservation, and governmental organisations resulted in a definition of wolf-dog hybrid for conservation and management. A summary document was developed that provides the context for the wolf-dog hybrid definition, and this document has been reviewed and published by the Large Carnivore Initiative for Europe (LCIE) (Stronen et al. 2025a). Subsequently, a perspective article building on the workshop document was published in a peer-reviewed journal (Stronen et al. 2025b).

3.1. Wolf-dog hybrid definition

Based on the Gelnhausen workshop discussion, we developed a wolf-dog hybrid definition, with further context and caveats detailed in Stronen et al. (2025a,b). The terms 'hybrid' and 'backcross' are not synonyms (see the Glossary in section 2). However, to be as simple and practical as possible, the wolf-dog hybrid definition below encompasses individuals that are first-generation hybrids (F1), as well as 1st and 2nd generation backcrosses to wolves (BC1w and BC2w).

Wolf-dog hybrid[2]: An individual with a level of dog admixture not less than the equivalent to the second-generation backcross to wolf (i.e. ≤ BC2w[3]; equivalent to having one dog as a great

[2] This wolf-dog hybrid definition is centred on a practical definition for conservation management, whereas the aims and temporal context of other fields, such as evolutionary research, might differ.

[3] *BC* stands for backcross, *2* for the number of generations, and *w* indicates that backcrossing occurred into the wolf parental population. Similarly, BC1w is a first-generation backcross into the parental wolf population, and these canids occur when a wolf reproduces with an F1-hybrid (wolf x dog offspring).

grandparent), which can be detected with high statistical confidence according to widely accepted, transparent molecular diagnostic procedures.

4. Standardisation of genetic analyses to detect wolf-dog hybrids in Europe

In Europe, genetic analyses to detect wolf-dog hybrids have been performed using different approaches, as detailed in Wolfness technical report D1 (Birkenhain et al. 2025). This review highlights the need for standardisation, also given the reconnection of European wolf populations (e.g. Hulva et al. 2018). We address key aspects such as genetic markers for individual identification and detection of population genetic structure, which are at various stages of integration across Europe, based, at least in part, on recent transboundary conservation projects such as LIFE WolfAlps EU (https://www.lifewolfalps.eu/, De Barba et al. 2024).

4.1. Genetic markers

Microsatellite or SNP-based genotyping may be used for WDH detection, if the chosen markers have been shown to be diagnostic (Birkenhain et al. 2025). Many laboratories genotype them using standard capillary electrophoresis instruments, although some now employ new high-throughput sequencing (HTS) microsatellite markers, where the results are standardised and can more easily be shared across laboratories (De Barba et al. 2024). Genotyping standardisation based on a panel of 22 HTS microsatellite markers has been achieved for the transboundary Alpine region as part of the LIFE WolfAlps EU project (https://www.lifewolfalps.eu/, De Barba et al. 2024). Analyses of samples from other European reference populations are also in progress with the HTS panel (Table 1). The results show that the current 22-HTS marker panel accurately identifies F1-hybrids and, based on a limited number of available profiles, it appears to also detect BC1w individuals. However, BC2w individuals identified by the 93-SNP panel developed by Harmoinen et al. (2021) to detect WDH at the European level have been inconsistently assigned with the 22 HTS markers. Thus,

the detection of BC2w canids requires analyses with the 93-SNP panel, or another (future) diagnostic panel.

Despite its limitations, capillary genotyping is also still used by several laboratories, and such analyses can play a key role in facilitating the inclusion of legacy data and genetic profiles from earlier years that offer valuable spatiotemporal context. For example, genetic profiles from capillary genotyping may be available from monitoring many years ago; however, if the DNA sources have been exhausted, it is not possible to re-genotype these samples using new methods. Genotyping newly collected samples with older marker panels allows researchers to compare such datasets from different time periods and assess the presence of individual animals across time.

If used alone, uniparental markers, including mitochondrial DNA (mtDNA) and Y-chromosome linked microsatellites, and coding markers, such as the K-locus (Caniglia et al. 2013; 2020), are insufficient for WDH detection. It is well known that genetically differentiated populations may share mtDNA haplotypes, which undermines the use of mtDNA markers for accurate population assignment (e.g. Wilson and Bernatchez 1998). Additionally, uniparentally inherited markers are blind to crossbreeding of parents who do not transmit this specific genetic information. For example, F1 hybrids resulting from a cross between a male dog and a female wolf carry only wolf mtDNA due to the exclusive maternal inheritance of this genetic material. The information provided by such markers may be traced back to generations of backcrosses that are too old to be of practical management concern for WDH, although this information can provide support and context for local monitoring and management (see also 4.2).

SNP markers are increasingly being used for the assessment of hybridisation, also in canids (Kraus et al. 2015**,** Eriksson et al. 2020, Harmoinen et al. 2021, Stronen et al. 2022, Delomas et al. 2023). A preliminary comparison between whole-genome profiles and results from the Harmoinen et al. (2021) panel indicated that the latter can be used to identify wolf-dog hybrids in Europe with a limited risk of error up to (and including) the BC2w. However, Italian and potentially Iberian populations require supplemental analyses, including the use of additional SNP markers, at least for the BC2w. Tests are ongoing with the complementary reduced panel

developed for the Italian, Iberian, and Dinaric populations, where the first two are highly divergent from other European wolves (Stronen et al. 2022), to clarify whether the inclusion of these additional SNPs will provide sufficient resolution for BC2w individuals. Finally, we are using whole-genome profiles genotyped within the Wolfness project to identify additional discriminant markers for wolf-dog hybrid detection (section 4.3).

4.2. Reference populations

In cases where assignment analyses have not included profiles from all relevant populations as references, dispersing wolves from genetically differentiated populations or their descendants may be incorrectly classified as having dog ancestry due to insufficient assignment probabilities to any wolf reference group. Care must be taken to correctly identify such dispersers, which are fundamental for maintaining functional connectivity among European wolf populations. Analyses of genetic data to detect WDH therefore require genotypes from surrounding wolf populations that are likely sources of dispersers. This approach permits us to distinguish divergent genetic profiles resulting from WDH (a concern) from those reflecting immigration from other wolf populations (generally deemed a benefit; Hindrikson et al. 2017). With this purpose, the Gelnhausen workshop participants identified 10 European wolf reference populations (Table 1), so that genetic laboratories can exchange genetic samples—or share genotypes for populations that have already been genotyped with the 22 HTS microsatellite panel (section 4.1)—to identify immigrants and their descendants.

Each reference population should include a minimum of 20 unrelated and non-admixed individuals to represent the genetic diversity of that population. We recommend that WDH analysis should include as reference all populations that may generate gene flow (i.e. be the source of dispersing individuals) and thus have the potential to confound local WDH assessments. It may also be necessary to include reference samples from dogs, especially if there are distinct and/or local dog breeds that may be expected to contribute to wolf-dog admixture. The reference populations in Table 1 are consistent with the most recent European wolf distribution map (Kaczensky et al. 2024) and population updates (Appendix 1).

However, in some cases, additional fine-scale spatial structure may be observed, whereby reference samples from specific areas are required for improved local resolution. One

example, and thus an exception to Kaczensky et al. (2024) and the map in Appendix 1, is the Dinaric-Balkan population, which has been listed as two separate reference populations for the purpose of this document. The reason is the presence of local genetic structure within Dinaric-Balkan region wolves (e.g. Šnjegota et al. 2021), which can influence the accuracy of WDH analyses (University of Ljubljana, unpublished data). Accordingly, wolves in Slovenia and Croatia have broadly been categorised as the Dinaric population, whereas wolves south and east of a transition zone centred in Bosnia & Herzegovina and southern Croatia (Šnjegota et al. 2021) are categorised as the Balkan population. Following the Gelnhausen workshop, a summary (Table 1) was prepared to assess the status of the identified reference populations, including whether samples have been genotyped for microsatellite markers using traditional capillary sequencing (CS) or, additionally, with the new HTS markers.

Ongoing gene flow among wolf populations requires assignment testing to verify individual ancestry, especially in transboundary areas (e.g. Hulva et al. 2018, Harmoinen et al. 2021, Konec et al. 2024). To limit the risk of including canids with recent dog ancestry, it is necessary to be conservative in defining the reference populations. Analyses with the 93-SNP panel by Harmoinen et al. (2021) to exclude any wolf-dog hybrids (i.e. F1, BC1w and BC2w, section 3.1) are therefore recommended. For the divergent Italian population, reference wolf profiles from Italy should also be included (Harmoinen et al. 2021), and additional uniparental markers (Y-linked microsatellites and mitochondrial DNA) and the K-locus associated with black coat colour have also been implemented (reviewed in Caniglia et al. 2020). The complementary panel from Stronen et al. (2022), which, together with the Harmoinen et al (2021), provides a total of 178 SNPs for divergent populations in southern Europe, can also be included to help screen out any individuals with signs of recent dog ancestry.

Table 1. European reference wolf populations – microsatellite genotyping status and sharing of reference population genotypes.

POPULATION	GENOTYPING STATUS[2]	NOTES ON SHARING OF REFERENCE POPULATION GENOTYPES
Alpine	CS/HTS	HTS reference genotypes in MBASE[3]
Balkan[1]	CS/HTS	HTS reference genotypes in MBASE[3]
Baltic	CS	
Carpathian	CS/HTS	HTS reference genotypes in MBASE[3]
Central European	CS/HTS	HTS reference genotypes in MBASE[3]
Dinaric[1]	CS/HTS	HTS reference genotypes in MBASE[3]
Italian Peninsula	CS	HTS reference: Work in progress at the University of Lausanne
Karelian	CS	
Iberian	CS	
Scandinavian	CS	

[1] Populations follow Kaczensky et al. (2024) except for the Dinaric-Balkan population, which has here been listed as two separate entities (see details in section 4.2).

[2] CS refers to capillary sequencing of microsatellites (traditional sequencing method), and HTS refers to high-throughput sequencing.

[3] Genotype profiles developed using HTS technologies are instantly comparable, and the MBASE database (https://github.com/divjalabs/mbase-genetics-wiki/wiki) will provide genetic profiles that can be used as wolf reference populations without the need for additional genotyping and calibration. [*The database is in progress, and genotypes are therefore in the development version of MBASE at present*.]

4.3. The current status of wolf-hybrid detection standardisation in Europe

To promote a standardised and reliable (i.e. objective, transparent, and repeatable) genotyping protocol for wolf-dog hybrid detection that is feasible across European wolf populations and widely adapted across European genetic laboratories, we propose a two-step approach:

A. Regional/national laboratories perform local analyses using their optimised markers and legacy data. These analyses need to consider the inclusion of all appropriate reference populations (section 4.2), including domestic dogs, wolves from local populations, wolves from other populations that may be expected to provide dispersers, and golden jackals.
B. In ambiguous cases, where hybridisation assessment is not successful in step A, the 93-SNP panel from Harmoinen et al. (2021), or another validated SNP panel for wolf-dog assignment, can provide additional resolution. The 93-SNP panel reduces the need for reference populations because this concern has been considered in its assembly, which was a central motivation for developing this Europe-wide array (Harmoinen et al. 2021). Previous analyses with microsatellite and SNP markers have nevertheless indicated that Italian wolves from the Alpine and Italian Peninsular populations are highly distinct (Stronen et al. 2013, Pilot et al. 2014, Montana et al. 2017, De Barba et al. 2024), and the inclusion of Italian reference samples improves assignments for these wolves (Harmoinen et al., 2021). Similar considerations may also apply to the Iberian wolf population, which diverged from a common ancestor at the same time as Italian wolves (Silva et al. 2020).

In parallel, within Wolfness, we are seeking to identify new discriminant markers for wolf-dog hybrid identification based on whole-genome sequencing. The Illumina CanineHD SNP panel used for the development of two earlier reduced SNP-panels for wolf-dog detection in Europe (Harmoinen et al. 2021, Stronen et al. 2022) has > 170,000 SNP markers. However, these SNPs were selected for dogs and may be missing important variation in wolves and other canids. Whole-genome sequencing data from wolves and admixed canids, including a wider range of

reference populations such as wolves from Türkiye, can therefore provide additional discriminant SNP markers.

4.4. Analytical approaches

Appropriate hybrid detection must involve the use of accepted sets of reference populations, commonly applied statistical tests involving Bayesian clustering/assignment methods (e.g. STRUCTURE, Pritchard et al. (2000); NewHybrids, Anderson and Thompson (2002); see also additional details in Birkenhain et al. (2025)). In the case of non-invasive samples, additional procedures, such as multiple replicates to determine the genotype of each sample must be followed to account for amplification errors that typically affect low-quality DNA sources (Taberlet et al. 1996, Waits and Paetkau 2025). Finally, in all analyses for detection of WDH, the exact methodology must be reported, and it should be clear from the study description (i) which reference samples have been included and (ii) whether these reference samples have previously been assigned to the population where they were collected. Furthermore, it can be informative to include a second method of analysis without any assumptions of genetic equilibrium conditions, typically an uninformed ordination such as principal component analysis (PCA) or other equivalent approaches. Such analyses can e.g. help detect outlier profiles that might represent dispersers from regions not included among the current reference populations, for example in the case of Russia and Belarus (Stronen et al. 2013, Pilot et al. 2014).

4.5. Thresholds for WDH assignment

In areas where hybridisation is known or expected to occur, it is advisable to perform analyses simulating the genetic profiles of hybrids and backcrosses up to and including BC2w using the relevant reference populations, especially when using microsatellite markers. These results can subsequently be used to establish local thresholds for WDH assignments (e.g. HybridLab, Nielsen et al. (2006)). Based on simulated genetic profiles of wolf-dog hybrids and backcrosses, the definition of probabilistic thresholds for hybrid classification can be informed through multi-event modelling when using a capture-recapture approach for the estimation of admixture prevalence (Santostasi et al. 2021).

Thresholds for WDH assignment should be based on the most reasonable compromise between Type I and Type II errors, reflecting the conservation status of a given wolf population, the history of admixture, and national laws and policies. The rationale for the chosen thresholds, the criteria used to define them, and the simulated confidence intervals for each hybrid class should be clearly explained. At present, an ancestry-informative SNP panel is the best available standard method for assessing hybrids up to BC2w (Harmoinen et al. 2021). Furthermore, given the rapid progress in sequencing platforms and tools for genomic analyses, we expect that analytical platforms and markers will be updated in the coming years. However, our primary aim with these guidelines is to promote the standardisation of analyses for detection of wolf-dog hybrids in Europe, based on the wolf-dog hybrid definition established at the Gelnhausen workshop (section 3.1). Therefore, we envision that updates to marker panels and analytical platforms will improve and further advance our initial efforts toward standardisation.

4.6. Additional considerations and concluding remarks

Increasing gene flow among wolf populations will augment genetic variability, and future monitoring efforts will need updated genetic tools, analytical pipelines and assumptions to track the amount and direction of gene flow (Caniglia et al. 2020). Furthermore, we are missing important information about gene flow and connectivity for wolf populations in Belarus and Russia, where increasing activities in erecting border fences are also expected to affect the movements of terrestrial wildlife (Nowak et al. 2024). Although these challenges are currently difficult to mitigate, it is important to acknowledge that different wildlife management approaches, including hunting (Jedrzejewski et al. 2010, Szewczyk et al. 2021) and gene flow from genetically differentiated populations (Stronen et al. 2013), might influence regional population structure and genetic diversity.

At present, we still have a limited understanding of the prevalence of wolf-dog hybrids across Europe. Many investigations are local or focused on areas where hybrids are known or expected to occur, and WDH prevalence estimates from such studies may, therefore, not be representative for the wider region (Santostasi et al. 2021). Accordingly, future spatial analyses, provided they are based on unbiased sampling (Wolfness technical report D3,

Ciucci et al. *in prep*), will provide key information to improve our understanding of the spatiotemporal dynamics of WDH in Europe and beyond.

Genetic and genomic analyses are in rapid development, and these guidelines provide a framework for standardisation of laboratory and analytical protocols to detect wolf-dog hybrids. They are based on our current knowledge and the genetic tools available at this time and therefore present a work-in-progress. We have highlighted certain ongoing developments that are expected to influence standardisation of analytical processes and protocols for the detection of wolf-dog hybrids, and also noted some future needs. These guidelines are focused on Europe, although further work is needed to test genetic marker panels developed and used in Europe to assess their performance further east. This encompasses (1) evaluation of the Harmoinen et al. (2021) panel of 93 SNP markers in Türkiye and the Caucasus, (2) development of additional discriminant markers for detection of canid hybrids based on whole genome profiles, and (3) improved understanding of wolf-dog hybrid ecology, including physiology and behaviour.

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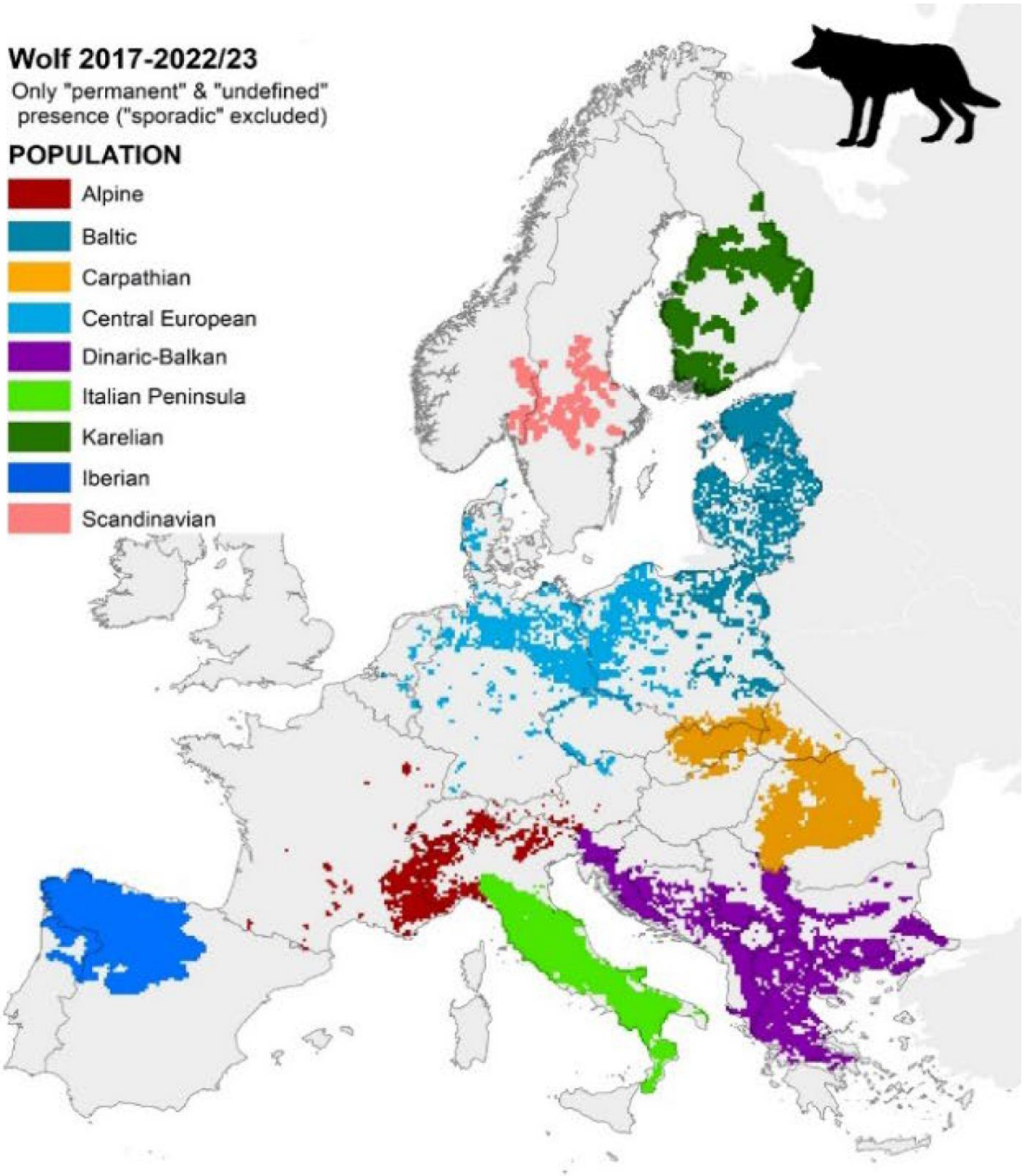
Appendix 1.



European wolf populations and their distributions based on data from 2017 to 2022/2023. Fig. 11, p. 35, in Kaczensky, P., Ranc, N., Hatlauf, J., Payne, J.C. et al. 2024. Large carnivore distribution maps and population updates 2017 – 2022/23. Report to the European Commission under contract N° 09.0201/2023/907799/SER/ENV.D.3 "Support for Coexistence with Large Carnivores", "B.4 Update of the distribution maps". IUCN/SSC Large Carnivore Initiative for Europe (LCIE) and Istituto di Ecologia Applicata (IEA). https://lciepub.nina.no/pdf/638672209981921829_2024_11_14_Large_Carnivore_range%20and%20population%20estimate%20update%202017-2022_1.2.pdf