Towards a formal definition and detection of wolf-dog hybrids to support policy and management at the EU scale[°][†]

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Introduction

Hybridisation (the interbreeding between individuals from genetically distinct lineages, regardless of their taxonomic status) with domestic or alien species presents an important threat for wild species. Such anthropogenic hybridisation occurs globally, across a wide range of species, and its extent is often underestimated [1]. The Biodiversa+ Wolfness project aims to address conservation impacts of anthropogenic hybridisation between the gray wolf (*Canis lupus*) and the domestic dog (*C. familiaris* or *C. l. familiaris* [2]) in Europe. While wolf-dog hybridisation (WDH) is a concern for long-term wolf conservation across the continent, the lack of consistent analytical protocols and hybrid definitions has so far limited research and conservation actions.

An authoritative pan-European policy statement, adopted in the framework of the Bern Convention on European Wildlife Conservation in 2014 (Recommendation No. 173 of the Standing Committee), currently recommends European countries to take action to address wolf-dog hybridisation, including to:

> "Take action to promote the detection of free-ranging wolf-dog hybrids, and to ensure government-controlled removal of detected wolf-dog hybrids from wild wolf populations;
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> Ensure that the government-controlled removal of wolf-dog hybrids takes place after government officials and/or the bodies entrusted by governments for this purpose and/or researchers have confirmed them as hybrids using genetic and/or morphological features. Removal should only be carried out by bodies entrusted by the competent authorities with such a responsibility, while ensuring that such removal does not undermine the conservation status of wolves."

The statement does not, however, define what is meant by a 'wolf-dog hybrid' [3].

In October 2023, the Wolfness project organised a workshop in Gelnhausen, Germany, with the aim of (*i*) reviewing laboratory protocols for wolf-dog hybrid identification and developing a standardised analytical protocol across Europe, and (*ii*) developing a shared definition of '*hybrid*' for conservation management. This document, which has been drafted by the workshop participants, and successively reviewed by members of the Large Carnivore Initiative for Europe (LCIE), summarises the key outcomes of the workshop. It is centred on the practical molecular aspects of wolf-dog hybrid detection. However, it is vital to note that obtaining unbiased estimates

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of admixture at the population level also requires proper *sampling designs* (Wolfness technical report in preparation).

1. RATIONALE

I. Why we need a definition of wolf-dog hybridisation (WDH)

To support management and legislation to the best of our knowledge and to effectively and timely preserve the genetic integrity of wolf populations.

As stated, the aim of our definition is to support legislation and management based on solid scientific grounds, but by no means is it intended to prescribe policy (i.e., to define management objectives in populations with wolf-dog interbreeding and how to implement conservation strategies to achieve them).

II. What are the properties of a wolf-dog hybrid definition

A definition should account for:

1. The intrinsic and technical limitations of current diagnostic procedures. That is, the understanding that a final assignment (i.e., detection of admixed individuals) is intrinsically probabilistic and uncertain, with the degree of uncertainty increasing with more generations since the hybridisation event (i.e., backcrosses, see General Definition of Terms below) and departure from robust and reliable detection procedures.
2. The realisation that admixture patterns in the wolf genome may be as old as domestication or may have occurred too far back in time to be of practical management concern. It follows that some limited extent of dog introgression must be regarded as part of the recent evolution of the wolf and can thus be tolerated.
3. Flexibility of management at the population level (responses may vary according to population size, trends, and extent of admixture).

III. Two fundamental questions

An effective definition cannot be simple and deterministic (e.g., based on rules of thumb or universally applied thresholds), and it requires answering some practical questions:

- What level of wolf-dog admixture should be considered to categorise an individual as a ‘hybrid’ for the purpose of conservation management?
- At what level can we reliably identify WDH using biological material typically collected during non-invasive genetic wolf monitoring?

2. GENERAL DEFINITION OF TERMS[‡]

[‡] Adapted from McFarlane & Pemberton (2019) Trends Ecol Evol 4, 315-326, DOI: 10.1016/j.tree.2018.12.013.

It is important that hybrid assignments are *reliable*, *comparable, replicable,* and *transparent* across Europe. This also requires standardised and unambiguous terminology and use of language for terms such as "hybridisation" and "introgression".

Hybridisation: mating of individuals from *genetically* diverged groups (including populations, species, etc.). When driven by human activities, this process is referred to as *anthropogenic* hybridisation.

Introgression: the incorporation of genetic variants from genetically diverged groups that is mediated by backcrossing of hybrids into the parental 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: an individual that has an intermediate genotype between two genetically diverged, parental groups as the result of recent interbreeding between individuals from these groups.

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" means the outcome of a mating between an F1 wolf-dog hybrid with a wolf, resulting in offspring with 75% wolf and 25% dog ancestry. A "second generation backcross to wolf" is the offspring of a first-generation backcross to wolf and a wolf (= 12.5% dog ancestry). While it is not its original meaning, recent backcrosses may be included in the term "hybrids" as well.

Hybrid swarm: an introgressed and hybridising population that comprises a large proportion of hybrids and backcrosses owing to a breakdown of mating barriers between two genetically diverged groups.

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).

3. PROPOSED DEFINITION

For use in conservation management[§] (i.e., to preserve the genetic integrity of wolf populations), we define a wolf-dog hybrid as an **individual with a level of dog admixture not less than the equivalent to the second-generation backcross to wolf** (≤ BC2w; i.e., a wolf whose great-grandparent was a dog), **that can be detected with high statistical confidence according to widely accepted, transparent molecular diagnostic procedures**. In other words, individuals

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[§] Here distinguished from the purposes of evolutionary research, where the study of hybridisation and introgression on a much broader timescale has informed our understanding of important selective processes such as immune function (e.g., Cubaynes et al. 2022, Science 378, 300-303, DOI: 10.1126/science.abi8745).

with less than 12.5% dog ancestry are not generally considered hybrids, although the caveats detailed below are integral to the definition.

Caveats

Although managers should be aware that this would increase the chance of Type I errors, the BC2w threshold may be extended to the equivalent of a third-generation backcrosses to wolves (i.e., BCw3), especially if supported by additional genetic evidence (e.g., uniparental and coding markers such as mitochondrial DNA (mtDNA) fragments, Y-chromosome linked microsatellites[**], and K-locus melanic deletion) and genetically confirmed anomalous phenotypic traits (see *Morphological considerations* below).

By referring to “transparent molecular diagnostic procedures” we mean that admixed individuals are identified based on a sufficiently large number of ancestry-informative markers, such as those provided by Harmoinen et al. (2021) [4], or published sets of microsatellites which (i) have been demonstrated to have a similarly high resolution for admixture detection based on simulated and real-world genotypes, and (ii) include comprehensive reference data from local and adjacent wolf populations within dispersal distance.

4. TOWARDS STANDARDISED MOLECULAR DIAGNOSTIC PROCEDURES ACROSS EUROPE

To promote a standardised, transparent, and repeatable genetic protocol that is feasible and widely adapted across Europe we propose a two-step protocol, where regional/national labs:

i. Perform local analyses using selected nuclear genetic markers and legacy data. These analyses also need to consider the inclusion of all appropriate reference populations, including domestic dogs (preferably local breeds and village dogs), local wolves, reference profiles from contiguous and potentially connected wolf populations that can be expected to provide immigrants, and related species such as golden jackals (*Canis aureus*);

ii. For profiles that cannot be clearly assigned to the above-mentioned reference profiles, we propose to use the 93-SNP panel from Harmoinen et al. (2021) [4] noted above or another SNP-panel which comprises those markers. This is especially relevant for backcrossed individuals, for which misidentification rates are likely to be higher. The panel from Stronen et al. (2022) [5] complements and adds to the resolution of the Harmoinen et al. [4] panel in the case of some genetically differentiated southern European wolf populations, especially of the Italian Peninsula (Caniglia et al. unpubl. data).

Reference populations

Wolf reference populations in assignment tests should comprise a minimum of 20-30 verified non-admixed individuals from each of the relevant wolf populations in Europe. Here, it is necessary to account for potential population substructure and wolf dispersal capacity [6], and all populations from which dispersers could arrive are therefore relevant for inclusion. In some cases, additional fine-scale genetic substructure may be observed, whereby reference samples from specific areas will be needed for improved local resolution. Those considerations are important to prevent that

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[**] Also known as short tandem repeats or STRs

dispersing wolves from genetically differentiated populations or their offspring are wrongly classified as hybrids due to low grades of genetic assignment to the local wolf population [4], and care must be taken to correctly identify such individuals. To prevent that wolves containing substantial dog ancestry are used as wolf references in molecular WDH testing, it is strongly recommended that reference samples have undergone appropriate testing, ideally with appropriate SNP panels or whole genome sequencing. Verified wolf reference samples should be exchanged among test laboratories to foster standardisation of WDH across Europe.

Important reference wolf populations are:

- Iberian
- Italian Peninsular
- Alpine
- Dinaric
- Balkan
- Carpathian
- Central European
- Baltic
- Karelian
- Scandinavian

Marker systems and laboratory analysis

As indicated above, an ancestry-informative SNP panel serves as the best available standard method to assess hybrids up to the BC2w generation. In cases with uncertainty regarding the individual ancestry, the SNP panel should be used to conduct comparative tests and calibrate locally applied microsatellite-based marker systems. However, given the rapid progress in sequencing platforms and tools for genomic analyses, we expect analytical platforms and individual markers to be adapted over time. Therefore, while our primary aim here is to agree on a definition and harmonise analyses, we acknowledge that markers and platforms may be updated in the future.

Both traditional microsatellite analysis based on fragment length analysis, and the emerging high-throughput sequencing of microsatellites, are appropriate tools for hybrid assessment if they have been calibrated using ancestry-informative SNP panels. However, uniparental and coding markers (mtDNA, Y-chromosome linked microsatellites, K-locus, Amylase copy number variation) are not sufficient, if used alone, for efficient WDH detection as they may trace back to generations of backcrosses too old to be of practical management concern or miss recent hybrids due to lack of resolution or uniparental inheritance.

In the case of non-invasively collected samples (e.g., scats or hairs), rigorous scientific standards, such as multiple analysis replicates (multiple-tube approach) must be followed to account for amplification errors and contamination. The fact that genotypes generated from non-invasive samples are error-prone due to low contents of often highly degraded DNA traces requires particularly cautious data interpretation, and strict data quality standards. Such analyses should always be performed by experienced laboratories with a solid publication record in non-invasive genetics.

Statistical Analyses and detection thresholds for hybrid classification

Appropriate hybrid detection must involve the use of verified sets of reference populations, commonly applied statistical tests involving Maximum-Likelihood (e.g., ADMIXTURE) or Bayesian (e.g., STRUCTURE, NewHybrids) clustering/assignment methods, and simulations to assess the discriminatory power of the marker panel (e.g., HybridLab). It is also informative to use a second method without any assumptions of genetic equilibrium conditions, typically an uninformed ordination such as a Principal Component Analysis (PCA) or other equivalent methods. The exact methodology for the analysis must be reported.

In areas where hybridisation is known or expected to be prevalent, it is advisable to perform simulations of hybrids and backcrosses from the above-mentioned relevant reference populations up to and including BC2w and use the results to establish local thresholds for wolf-dog hybrid assignments. In capture-recapture applications, a multi-event model formulation allows assessment of uncertainty in hybrid classification based on simulated genotypes[7]. Assignment thresholds used to classify admixed individuals should be based on the most reasonable compromise between the risk of erroneously identifying a wolf as a hybrid (i.e., Type I error) and the risk of erroneously identifying a hybrid as a wolf (i.e., Type II error), and not only on the former, as both have relevant implications management-wise. Specifically, the latter can be particularly harmful in small and increasing wolf populations or by negating prompt management interventions to prevent hybridisation in local packs from spreading further. The rationale for the thresholds, the criteria used to define them, and the simulated confidence intervals for each hybrid class should be clearly stated, as these are relevant for managers to define context-specific management policies.

5. USEFULNESS OF MORPHOLOGICAL TRAITS

Genetic indicators of hybridisation are not always associated with objectively definable and noticeable atypical morphology, resulting in morphologically *cryptic* hybrids. On the other hand, morphological traits considered by some to be anomalous may represent intraspecific variation, traces of introgression too old to be of practical management concern, or reflect subjective evaluation, especially if they have not been genetically supported. Hence, if used alone, morphological cues are often neither reliable nor objective evidence of hybridisation. For conservation management, we recommend that allegedly anomalous morphological cues detected through live-trapping, direct observation, camera trapping, wolves culled or otherwise found dead be routinely confirmed through *ad-hoc* genetic analysis. Over time, and if fostered by genomic approaches, these data will contribute vital information on those morphological traits that may be valuable indicators of WDH.

6. PRACTICAL CONSIDERATIONS

It could be argued that identifying wolf-dog hybrids uniquely by genetic means is not a viable option to implement timely management responses, as the time and effort usually required to obtain genetic evidence of hybridisation may be incompatible with the need for timely management decisions about wolf-dog hybrids. Likewise, concerns may be expressed that the prescriptive adoption of genetic evidence to identify wolf-dog hybrids may be used as a cover for inaction by regional or national administrations that encounter public opposition to the removal of wolf-dog hybrids. In contrast, morphological cues of hybridisation would be easily obtainable through camera trapping or direct observations and would immediately inform management interventions. Moreover, it may also be noted that, even at the cost of a higher Type I error (see section General Definition of Terms) timely management responses would be preferable to inaction to prevent the development of a hybrid swarm.

In the light of the above concerns, it is beneficial to further clarify that our wolf-dog hybrid definition is not *de facto* intending to be policy prescriptive (see point 1.I above). Management decisions should be made by managers and administrators based on the best science available but also on practical considerations that apply to specific wolf populations and their ecological and social context. For instance, whether morphological cues should be used or not as evidence of hybridisation for practical management goals depends on how the two types of error in detecting hybrids (i.e., erroneously identify a wolf as a hybrid versus erroneously identifying a hybrid as a wolf) are thought to affect the conservation of a given wolf population [8]. With reference to recent legislation at the European level, it is worth recalling that Recommendation no. 173 adopted by the Standing Committee of the Bern Convention recognises anomalous morphological traits as cues of hybridisation (see also Introduction), even though the European Commission guidance document on the application of the Habitats Directive fully recognises the “difficulty of distinguishing hybrids from genetically ‘pure’ wolves based only on morphological characteristics”. Our findings based on genetics and genomics confirms this last point.

The latter document also requires management plans addressing wolf-dog hybridisation to be based on “fully described and justified” criteria. In our view, justification of a given criterion (e.g., how to identify wolf-dog hybrids for management purposes) can be seen as a two-step process, where a strictly science-based definition first informs the process, and then its practical implications are discussed, adjusted, and adopted based on the specific context and wolf population. In this document, we thus purposely limited our aim to provide a definition of wolf-dog hybrids based on the best available science. Our definition, if eventually endorsed by the competent authorities, should be discussed in terms of its practical implications by other actors (i.e., decision makers, administrators, managers) who have the responsibility to design and implement management policies, conservation strategies, and justify the therein adopted criteria.

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