Title page:

Practical genetic diversity protection: an accessible framework for IUCN subpopulation and Evolutionarily Significant Unit identification

Authors: Geue J.C*., Bertola L.D., Bloomer P., Brüniche-Olsen A., da Silva J.M., DeWoody J.A., Fedorca A., Godoy J. A., Grueber C.E., Hunter M.E.*, Hvilsom C., Jensen E.L., Kopatz A., MacDonald A.J., Pérez-Espona S., Piaggio A.J., Pierson J., Russo IM.*, Senn H., Segelbacher G.*, Sunnucks P., van Oosterhout C., Leigh D.M*.

*co-corresponding author

Julia C. Geue

julia.geue@wildlife.uni-freiburg.de

1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland

2 Wildlife Ecology and Management, University Freiburg, Freiburg im Breisgau, Germany

ORCID:0000-0002-1038-8614

Laura D. Bertola

laura.bertola@gmail.com

1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland

2 Department of Biology, University of Copenhagen, Copenhagen, Denmark

3 National Centre for Biological Sciences, TIFR, Bangalore, India.

ORCID: 0000-0002-3445-0355

Paulette Bloomer

paulette.bloomer@up.ac.za

1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland

2 Molecular Ecology and Evolution Programme, Department of Biochemistry, Genetics and Microbiology, University of Pretoria, Pretoria, South Africa

ORCID: 0000-0002-6357-0153

Anna Brüniche-Olsen

anna.bruniche-olsen@sund.ku.dk

1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Center for Macroecology, Evolution and Climate, GLOBE Institute, University of Copenhagen, Copenhagen, Denmark
ORCID: 0000-0002-3364-2064

Jessica M. da Silva
j.dasilva@sanbi.org.za
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 South African National Biodiversity Institute, Kirstenbosch Research Centre, Cape Town, South Africa
3 Centre for Ecological Genomics and Wildlife Conservation, Department of Zoology, University of Johannesburg, South Africa
ORCID: 0000-0001-8385-1166.

J. Andrew DeWoody
dewoody@purdue.edu
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Depts. of Forestry & Natural Resources and Biological Sciences, Purdue University, West Lafayette Indiana, USA
ORCID: 0000-0002-7315-5631

Ancuta Fedorca
ancutacotovelea@yahoo.com
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Department of Wildlife, National Institute for Research and Development in Forestry "Marin Dracea", Brasov, Romania
3 Department of Silviculture, Faculty of Silviculture and Forest Engineering, Transilvania University of Brasov, Brasov, Romania
ORCID: 0000-0001-5828-5422

José A. Godoy
godoy@ebd.csic.es

1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Estación Biológica de Doñana, CSIC, Sevillle, Spain
ORCID: 0000-0001-7502-9471

Catherine E. Grueber
catherine.grueber@sydney.edu.au
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 School of Life and Environmental Sciences, Faculty of Science, The University of Sydney, Camperdown, Australia
ORCID: 0000-0002-8179-1822

Margaret E. Hunter
mhunter@usgs.gov
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 U.S. Geological Survey, Wetland and Aquatic Research Center, Gainesville, Florida, United States of America
ORCID: 0000-0002-4760-9302

Christina Hvilsom
ch@zoo.dk
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Copenhagen Zoo, Frederiksberg, Denmark
ORCID: 0000-0001-7870-6888

Evelyn L. Jensen
evelyn.jensen@newcastle.ac.uk
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 School of Natural and Environmental Sciences, Newcastle University, Newcastle Upon Tyne, UK
ORCID:0000-0002-1819-3117

Alexander Kopatz
alexander.kopatz@nina.no

1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Norwegian Institute for Nature Research, Trondheim, Norway
ORCID: 0000-0002-9139-1179

Anna J. MacDonald
anna.macdonald@aad.gov.au
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Australian Antarctic Division, Department of Climate Change, Energy, the Environment and Water, Kingston, Australia
ORCID: 0000-0003-2972-200X

Silvia Pérez-Espona
silvia.perez-espona@ed.ac.uk
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Royal (Dick) School of Veterinary Studies and The Rosline Institute, Edinburgh, United Kingdom
ORCID: 0000-0001-7098-4904

Antoinette J. Piaggio
Toni.J.Piaggio@usda.gov
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 United States Department of Agriculture, Animal Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado, United States of America
ORCID:0000-0002-4701-0746

Jennifer C. Pierson
jennifer.pierson@australianwildlife.org
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Australian Wildlife Conservancy, Subiaco, WA, 6008, Australia
ORCID: 0000-0003-4140-010X

Isa-Rita M. Russo
russoim@cardiff.ac.uk

1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 School of Biosciences, Sir Martin Evans Building, Cardiff University, Wales, United Kingdom
ORCID: 0000-0001-9504-3633

Helen Senn
hsenn@rzss.org.uk
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Royal Zoological Society of Scotland, Edinburgh Zoo and Highland Wildlife Park, Scotland, UK
ORCID: 0000-0002-3711-8753

Gernot Segelbacher
gernot.segelbacher@wildlife.uni-freiburg.de
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Wildlife Ecology and Management, University Freiburg, Freiburg im Breisgau, Germany
ORCID: 0000-0002-8024-7008

Paul Sunnucks
paul.sunnucks@monash.edu
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 Wildlife Genetic Management Group, School of Biological Sciences, Monash University, Melbourne 3800, Victoria, Australia
ORCID: 0000-0002-8139-7059

Cock van Oosterhout
C.Van-Oosterhout@uea.ac.uk
1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland
2 School of Environmental Sciences, University of East Anglia: Norwich, Norfolk, GB
ORCID: 0000-0002-5653-738X

Deborah M. Leigh
deborah.leigh@senckenberg.de

1 International Union for Conservation of Nature (IUCN) Conservation Genetics Specialist Group (CGSG), Gland, Switzerland

2 LOEWE Centre for Translational Biodiversity Genomics, Frankfurt, Germany

3 Senckenberg Research Institute, Frankfurt, Germany

4 Institute of Ecology, Evolution, and Diversity, Faculty of Biosciences, Goethe University Frankfurt, Frankfurt, Germany

ORCID: 0000-0003-3902-2568

Abstract

The International Union for Conservation of Nature (IUCN) sets global conservation standards, including the Red List of Threatened Species and the Green Status of Species. Recent analyses showed that genetic diversity has not been effectively considered by IUCN species assessments, despite being fundamental to species' fitness and adaptive potential. Incorporation of genetic diversity into IUCN assessments can support its successful long-term conservation. To enhance the preservation of genetic diversity, assessments should include genetically meaningful within-species units. Subpopulations are recognized units by the IUCN for protecting natural connectivity, however infrequently evaluated. Evolutionarily Significant Units (ESUs) are currently not recognized as a formal unit by the IUCN. However, incorporating ESUs into conservation frameworks could significantly enhance our capacity to identify and protect adaptive genetic diversity. To facilitate inclusion of these units in IUCN assessments, we outline a widely applicable framework for their identification that uses non-molecular and molecular data for global accessibility.

Keywords

Conservation policy

Evolutionarily Significant Unit

Genetic diversity

IUCN species assessments

Subpopulations

Main text

Key terms

Subpopulations in the International Union for Conservation of Nature (IUCN) Red List framework are geographically or otherwise distinct groups in a population between which there is little demographic or genetic exchange (typically one successful migrant individual or gamete per year or less (IUCN, 2024a); . Subpopulations are similar to the population unit used for genetic diversity monitoring within the CBD Kunming-Montreal Global Biodiversity Framework (CBD, 2022; Hoban et al., 2020). However, the term population has a very different meaning within the IUCN framework (see below).

A population within the IUCN Red List framework is the total number of individuals in a taxon. This can encompass an entire species or refer only to a subspecies (IUCN, 2024a). Population size refers only to mature individuals in IUCN assessments. Due to the multiple definitions of this term, we have avoided it in the remainder of this manuscript.

Evolutionarily Significant Units "ESUs" are lineages demonstrating highly restricted gene flow from other such lineages within the higher organizational level (lineage) of species (Fraser & Bernatchez, 2001). Due to limited gene flow, these subpopulation networks follow their own evolutionary trajectory and thus house unique adaptive genetic diversity (Funk et al., 2012).

Allelic diversity is a measure of genetic diversity and refers to the number of different alleles, variants of DNA sequences at a specific location on the genome—i.e., locus—in a group of individuals.

Heterozygosity is the proportion of individuals with different alleles at a locus. Heterozygosity is often reported as an average (across loci or individuals) within a genetic unit (e.g., subpopulation) or sampling location.

Why list Subpopulations and Evolutionarily Significant Units in IUCN assessments?

Genetic diversity underpins fitness, resilience, and adaptive potential (Hughes et al., 2008; Meek et al., 2023; Reed & Frankham, 2003) and is closely linked with species extinction risk. Though understanding of genetic diversity's role in the resilience of wild species and their ecosystems goes back to the foundation of evolutionary thought (e.g., Charles Darwin's "tangled bank" in the Origin of Species 1859), it was not until 2022 that protection of genetic diversity in wild species was comprehensively included in global conservation targets (Target 4 of the UN Convention on Biological Diversity Kunming-Montreal Global Biodiversity Framework) (CBD, 2022). However, genetic diversity remains rarely considered in other global conservation programs (e.g., neglected in protected area design (Paz-Vinas et al., 2023),

species recovery plans (Pierson et al., 2016), in IUCN assessments (Schmidt et al., 2023), and in recent policy like the European Union Nature Restoration Law (O'Brien et al., 2024)).

Genetic diversity is in decline globally, with irreversible allelic diversity and heterozygosity loss recorded in wild species (Exposito-Alonso et al., 2022; Leigh et al., 2019; Shaw et al., 2025). Human activities are frequently leading to the extirpation of entire subpopulation networks (Ceballos et al., 2017; Mastretta-Yanes et al., 2024), which likely harboured local adaptations and distinct alleles, thus representing loss of unique evolutionary trajectories. The full effects and extent of genomic erosion are difficult to assess because they are not always instant (Pinto et al., 2024). For example, impacts on fitness may become realized only during environmental change (Gargiulo et al., 2024). Nevertheless, it is clear that genetic diversity loss is decreasing species fitness, reducing adaptive potential and the capacity to respond to change. We are in an era of rapid environmental change where species must adapt to persist, and enhancing the integration of genetic diversity into global conservation programs and policies can improve species persistence.

The International Union for Conservation of Nature (IUCN) and its Red List (a catalogue of over 163,000 species) is the international standard in assessing the extinction risk of species. Extinction risk is calculated based on the best available information on occupation of the focal species' historical range, census size and trends, habitat quality and fragmentation level (IUCN, 2024a); genetic diversity is not explicitly considered. In the more recently established IUCN Green Status of Species (IUCN, 2021), the recovery to species census size observed prior to major human impacts is assessed as a measure of conservation success. Genetic diversity is also not yet explicitly considered, but is present in the text of some Green Status assessments (e.g., Alpine ibex; (Brambilla et al., 2020).

Due to the omission of genetic diversity from assessment criteria, there is no clear linear relationship between levels of genetic diversity and IUCN conservation status (Red List extinction risk category) (Brüniche-Olsen et al., 2021; Díez-del-Molino et al., 2018; Jeon et al., 2024). Species across each IUCN threat category show signs of loss of genetic diversity (Leigh et al., 2019; Mastretta-Yanes et al., 2024; Shaw et al., 2025). Furthermore, species that have undergone profound, irreversible genetic diversity loss have received high Green Status recovery scores, despite persistent risk of inbreeding and limited adaptive capacity (e.g., Alpine Ibex; Brambilla et al., 2020). The consequences of this mismatch are that species' extinction

risk is often underestimated, likely fuelling suboptimal conservation management decisions (Jeon et al., 2024; Schmidt et al., 2023).

The lack of genetic diversity inclusion in IUCN assessment criteria represents a challenging oversight to resolve (Schmidt et al., 2023; van Oosterhout, 2024). Globally, <1.5% of species on the IUCN Red List have publicly accessible nuclear genetic or genomic data (Paz-Vinas et al., 2023). Consequently, directly incorporating molecularly-derived genetic diversity metrics into IUCN assessments is not widely implementable at this point. However, this does not mean that steps to improve genetic diversity safeguarding are unattainable. The recently adopted genetic indicators in the Kunming-Montreal Global Monitoring Framework (GBF) provide equitable access to genetic diversity protection without necessarily requiring molecularly-derived information (Hoban et al., 2025; Mastretta-Yanes et al., 2024). These indicators focus on maintaining genetic diversity through protection of evolutionary processes. Here we propose to apply a similar approach into IUCN assessments to improve genetic diversity protection in a globally accessible manner.

Conservation-relevant evolutionary genetic processes can be categorized into two spatiotemporal scales (Figure 1). First, short-term processes (gene flow and genetic drift) shape the genetic structure of species over tens of generations across subpopulations. Second, longer-term processes of environmental adaptation (i.e., natural selection) and the accumulation of alleles through mutation shape the evolutionary trajectory of a species across distinct networks of several subpopulations. These latter distinct networks are “Evolutionarily Significant Units” (hereafter ESUs) (Allendorf et al., 2022). Protecting subpopulations and ESUs within IUCN assessments could help to protect evolutionary genetic processes, representing a significant step towards genetic diversity safeguarding. Notably, ESUs are demographically independent within-species units and as a separate considered level also offers a more accurate representation of extinction risk. While an entire species may have a healthy census size, natural ESU subdivisions will translate to much smaller demographic units. A species with five ESUs could have a total census size of 10 thousand and be considered low risk, but within that there could be ESUs with 10s or 100s of individuals, which are at very high risk of extinction.

A history of dividing species into Evolutionarily Significant Units:

While IUCN-defined subpopulations are unique to the assessment framework, ESUs have a longstanding history in conservation genetics. ESUs generally represent a single subpopulation or a network of subpopulations with high genetic and ecological distinctiveness meriting protection (Crandall et al., 2000; Funk et al., 2012; Moritz, 1994; Robertson et al., 2014). The term ESU was first conceptualized by Ryder (1986) as a subset of unique genetic attributes within a species important for present and future generations. In order to identify such unique genetic attributes and delineate ESUs within species, Ryder (1986) suggested using a combination of genetic data, geographic distribution data, life history information and morphometrics.

In subsequent years, others proposed varying definitions and criteria to identify ESUs and separated species into conservation-relevant units (overview in Fraser & Bernatchez, 2001). ESU concepts and delineations ranged from reproductive isolation (based on e.g., life history patterns, genetic structure, habitat occupancy; Waples, 1991) to reciprocal monophyly based on mitochondrial DNA and significant divergence of allele frequencies (Moritz, 1994). With the era of genomics, definitions were adapted to genomic measures to enable a more direct assessment of adaptive genetic diversity (e.g., Funk et al. 2012). Conflicting views arose about the use of genetics alone to separate species into manageable conservation units (e.g., Moritz, 1994). Ecological factors, such as habitat specialization or behavioral differences, play a role in the significance of subpopulation networks and might be overlooked if genomic data alone are used to determine ESUs. A more holistic approach is to use genetic and non-genetic metrics depicting adaptive diversity and other evolutionary processes to identify ESUs (e.g., Crandall et al. 2000; de Guia & Saitoh 2007; Fraser & Bernatchez 2001; Funk et al. 2012; Robertson et al. 2014). Considerable challenges around the term and use of ESUs range from debates over defining criteria or 'frameworks', to broader implications for conservation policy and resource allocation. Subjectivity in defining criteria for ESUs (e.g., genetic vs. ecological ones) is a longstanding issue that has resulted in a lack of standardization in the term's application. Standardization has thus far failed due to the multidisciplinary nature of conservation science, and because scientists from different disciplines have seen value in defining and using ESUs with different approaches and underlying data-types. These inconsistencies limit cross-comparability, impacting

conservation prioritizing efforts and value assessments (Allendorf et al., 2022; Robertson et al., 2014), but could be resolved through the acceptance of standardized frameworks.

Despite their complex history, ESUs represent units with unique genetic characteristics whose protection can help to ensure the genetic diversity of a species (or multiple species; Black et al. 2024). ESUs also have their own evolutionary trajectory and may occupy unique or different ecological niches to other units within their species. Thus, protecting ESUs helps maintain evolutionary processes beyond the unit and species by supporting ecosystem function.

The IUCN Red List currently assesses species' extinction risk at three levels: entire species, subspecies, and subpopulations. Subspecies are scientifically controversial for many reasons (Starrett, 1958), but the most relevant is that they often fail to reflect within species genetic units (e.g., Cape parrot, Coetzer et al., 2015); Leopard skink, Prates et al., 2023). While subpopulations can reflect important units for genetic diversity protection, less than 5% of species have subpopulations on the IUCN Red list (Janet Scott, Programme Officer at IUCN, written communication, April 2024) because their identification remains unstandardized.

Here, we describe how the incorporation of ESUs into IUCN listing level can better reflect within-species units and protect adaptive processes. To aid their inclusion, we have developed a comprehensive and flexible framework for standardized ESU identification. We also propose a standardized methodology that can be applied to improve subpopulation identification in IUCN-based species assessments (Red List and Green Status) and greater genetic diversity safeguarding. Integrating ESUs into the Red List would be a long-term effort, requiring additions to the assessment criteria that we outline at the end of this article, but could be more readily built into Green Status development.

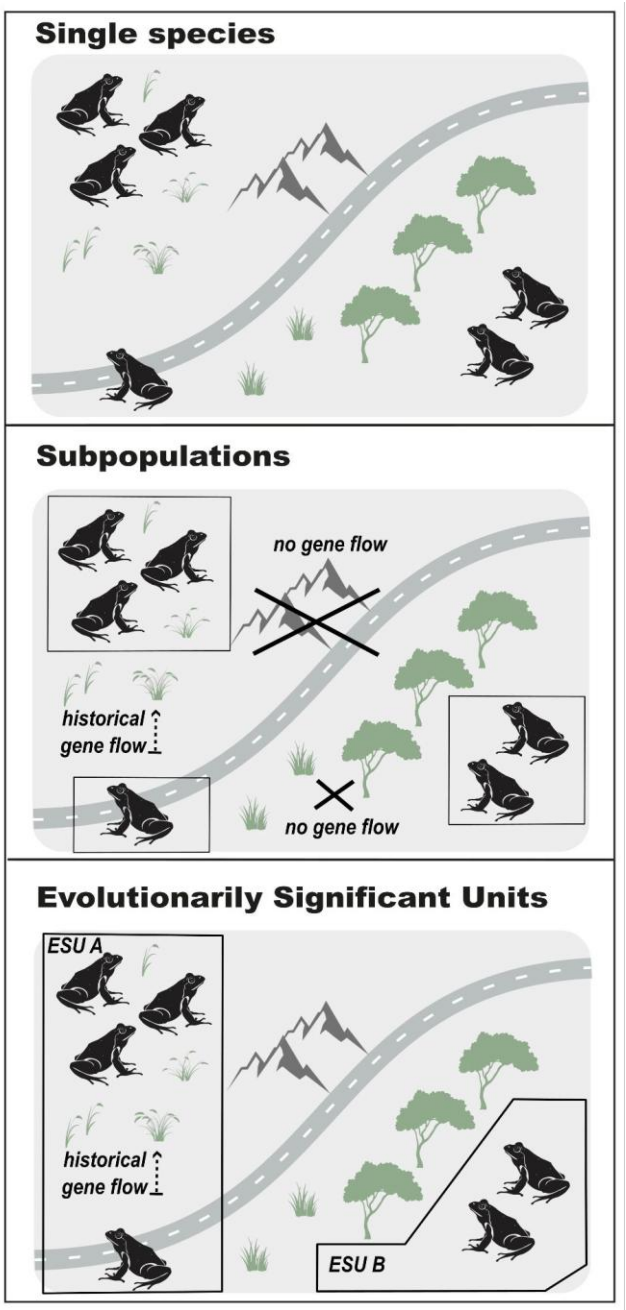

Figure 1 - Within the IUCN assessment guidelines, listing of distinct groups can occur at species- (top panel), subspecies- (not shown), or subpopulation (middle panel) levels. We suggest the inclusion of Evolutionarily Significant Units "ESUs" (bottom panel). Note that one or several subpopulations together can form an ESU. The current definition of subpopulation (and subspecies) includes situations where there is a wide range in the level of gene flow between the proposed groupings, from none to substantial. The addition of an ESU category would create a division only when gene flow is highly restricted or absent. In this cartoon example of a fictional frog species, gene flow is restricted by geographical barriers (e.g., mountains), but in reality, natural gene flow restriction can be created by a wide variety of factors.

Subpopulation and ESU standardized framework

The framework that we outline for standardized identification of subpopulations and ESUs has two steps: first, identifying genetically meaningful subpopulations and, second, grouping these into ESUs. Though we recognize the existence of several ESU frameworks (e.g., Fraser & Bernatchez, 2001; Funk et al., 2012), a framework using multiple lines of evidence is necessary for objective and standardized identification of IUCN-relevant subpopulations.

Three types of data are used in this delineation framework: *genetic*, *recorded biological* and *inferred* evidence (Figure 2A). *Genetic* evidence derives from genetic or genomic markers (e.g., single nucleotide polymorphisms [SNPs], microsatellites, mitochondrial haplotypes) and is leveraged to assess differentiation and/or evolutionary distinctiveness of groups within a species. *Recorded biological* evidence does not require genetic data (e.g., observed biogeographic patterns, variation in transmitted traits) and comes directly from individuals in the assessed focal species. *Inferred* evidence also does not require genetic data and is not directly observed in the focal units, e.g., is deduced from biogeographic patterns based on modelling techniques. To guide assessors, we have comprehensively listed categories, specific analyses within each evidence-type and data that can be used (Table 1).

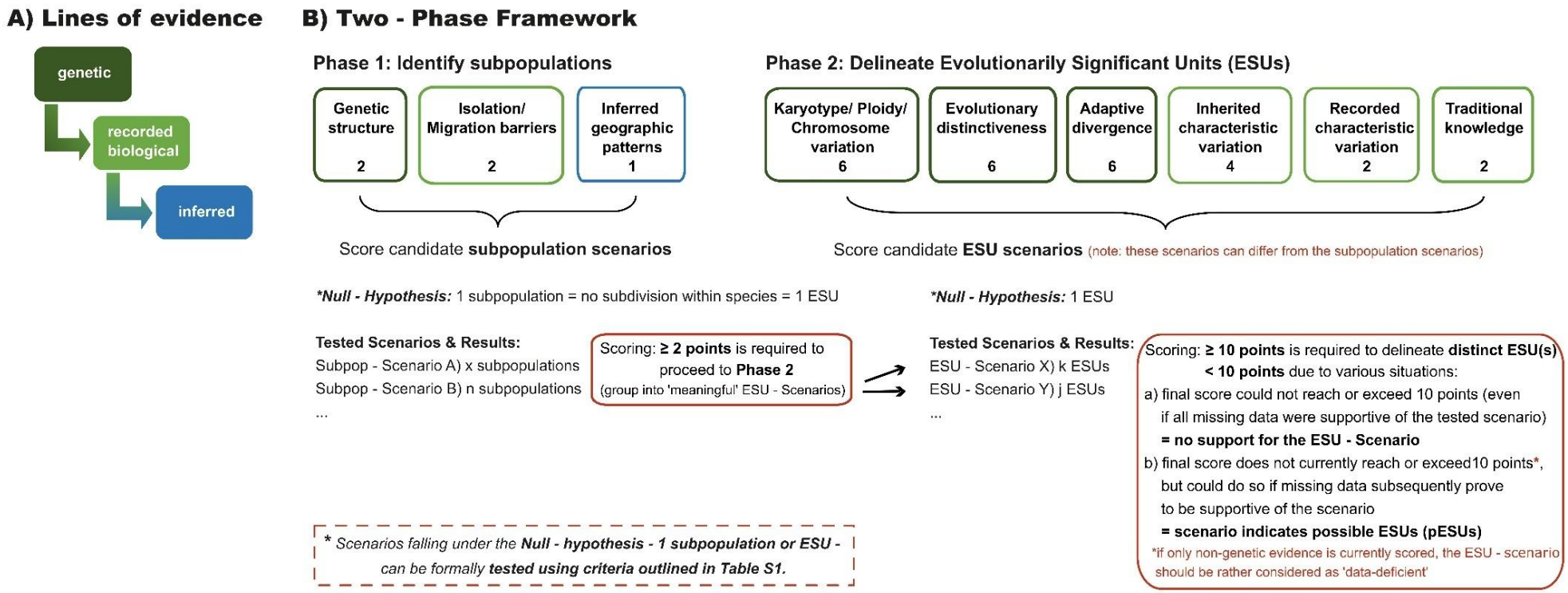

Figure 2 - Lines of evidence A) and the Two-phase framework B) to assess the strength of evidence for subpopulations and Evolutionarily Significant Units (ESUs). Different lines of evidence are highlighted in colour (dark green, green and blue). Scores for each line of evidence are indicated in the boxes and reflect the informative value of each line of evidence to the likelihood of subpopulations and ESU(s) being distinct, with higher scores indicating increased support. The framework is worked in candidate 'scenarios', determined by conflicting evidence of the presence of subpopulations for the assessed species. Each scenario should be scored separately.

345 *Table 1 - Lines of evidence used in the two-step framework to delineate subpopulations and ESUs.*
346 *For each line of evidence, a description and example methods to assess lines of evidence are*
347 *described. For each line of evidence example studies and the score given are mentioned.*
348

Types of evidence	Description	Example methods of assessments	Example studies using lines of evidence*	Score
Genetic evidence				
Genetic structure *(Phase 1)*	Substantial genetic structure and fragmentation mostly determined by limited gene flow between subpopulations.	Structure-like analyses, differentiation statistics (pairwise Fst values), private alleles within subpopulations.	e.g., Abbott & Double, 2003	2
Karyotype, ploidy, and chromosome structure variation *(Phase 2)*	Documented heritable differences in chromosome number, ploidy, or chromosome structure between candidate ESUs.	Evidence of heritable differences from cytogenetic analysis and structural variant detection software and/or evidence of no/rare/unfit hybrids between proposed units.	e.g., Ahrens et al., 2020; Ferreira et al., 2017; Hollenbeck et al., 2022	6
Evolutionary distinctiveness *(Phase 2)*	Genetic evidence of reproductive isolation, due to highly restricted or no gene flow between focal ESUs that may have fuelled evolutionary distinctiveness.	Long evolutionary divergence times and/or reciprocal monophyly. In complex cases follow statistical support (above 75% support on branches that define a clade), derived from a statistically valid evolutionary model and tree-building approach.	e.g., Moritz, 1994; Moritz & Faith, 1998; Walsh et al., 2024	6
Adaptive divergence *(Phase 2)*	Robust genetic evidence of candidate ESUs harbouring unique local adaptation(s) driven by selection (e.g., environmental, sexual selection) that are not shared with other ESUs.	Robust genomic signals of local adaptations or stable hybrid zones.	e.g., Bonin et al., 2007; Rodríguez-Quilón et al., 2016	6

Recorded biological evidence

Isolation and barriers to migration *(Phase 1)*	Subpopulations show evidence of isolation (i.e., long-term signs of restricted gene flow due to geographic, environmental, or temporal differences) between them. Alternatively, subpopulations show evidence of recently restricted gene flow due to human mediated change (e.g., habitat fragmentation, extirpation of connecting subpopulations etc).	Occupation of different biogeographical zones. Occupation of discrete remnants of historical habitats with little to no chance of natural migration between habitat patches.	e.g., Hewitt, 2004; Lorenzen et al., 2012	2
Inherited characteristic variation *(Phase 2)*	Candidate ESUs show consistent heritable differences in life history or ecologically/species important traits (e.g., body size, colour, breeding time, use of spawning grounds).	Heritable differences in focal traits observed in common garden experiments or other robust analytical tests (e.g., cross fostering).	e.g., Small et al., 1998; Wainwright et al., 2008	4
Recorded characteristic variation *(Phase 2)*	Candidate ESUs show consistent differences in traits that are locally transmitted but not robustly shown to be heritable (acquired behavioural or phenotypic differences; environmentally modified traits).	Traits differ between units but may be acquired or transmitted (non-genetic). For example, cultural and/or learnt behavioural differences like foraging techniques, alternative migration routes, regional birdsong 'dialects'; methylation differences; body size differences).	e.g., Gu et al., 2021; Lundberg et al., 2017; Sanchez-Donoso et al., 2022; Toews et al., 2019	2

Traditional knowledge *(Phase 2)*	Distinctiveness between candidate ESUs based on indigenous, local, or traditional knowledge.	Recorded information that stems from indigenous or local knowledge (according to the IUCN ILK framework, IUCN, 2022). This encompasses information that is not yet statistically analysed, information that is analysed should be counted as inherited or recorded characteristics.	2
Inferred evidence			
Inferred geographic patterns *(Phase 1)*	Subpopulation differentiation and fragmentation modelled from the focal species through e.g., species distribution modelling approaches, or measured/ observed from closely related species.	Subpopulation disjunction, phylogeographic and/or biogeographic evidence from co-occurring species used as a proxy, assumption of biogeographical data/refugia without direct evidence, dispersal distances and buffer (also used in the CBD genetic indicators, Hoban et al., 2020).	1

To offer a clear threshold for delimiting subpopulations or ESUs, each line of evidence is given a score. The value of the score corresponds to the strength of evidence. Scores range from 1 to 6, values of 6 are for evidence-types that offer the strongest support (i.e., genetic/genomic signals of prolonged reproductive isolation and/or local adaptations). A score of 6 is only possible in Phase 2 as these differences occur between ESUs. Scores of 4 and 2 are given to strongly suggestive lines of evidence that reflect genetic divergence or non-molecular signs of local adaptation. A score of 1 is given to data that are entirely inferred, such as information from projections (e.g., maps, species distribution models) or information inferred from closely-related species. This is a common, but high-risk evidence-type, and its low score reflects this associated risk. The relative weighting of evidence-types reflects the IUCN's nature of evidence rule for assessment criteria (see IUCN, 2024a). The framework does not allow for fractions or partial scores, scores are in even increments to support quick summing and development of the tool over time. We have strived to keep tallying the scores as simple as possible, but a degree of complexity is needed to capture all outcomes.

To determine the number of divisions, assessors should leverage the collected evidence from the categories detailed in Table 1 and divide the species into the most likely groups of subpopulations and ESUs, each grouping is called a candidate 'scenario'. In the case of conflicting evidence, assessors should divide the species into all different 'scenarios' and score each scenario separately. Different scenarios can be tested for Phase 1 and 2 and subpopulations can be grouped into a smaller number of ESUs. Comprehensive testing of scenarios based on existing knowledge, covering e.g., 1-7 subpopulations or ESUs is unnecessary, as many will be lacking sufficient data. The results of the scenario with the highest score (note this may be different for ESUs and subpopulations) could be used for listing assessment. When defining subpopulations please note that subpopulations can also be driven by human-mediated fragmentation or disruption of gene flow, but ESU divisions are likely to be more ancient.

To identify distinct subpopulations a total score of ≥2 is required in Phase 1. If multiple scenarios are tested, the scenario with the highest score is used. If scores are tied, we recommend choosing the high-score scenario that meets the needs for species management. If two or more distinct subpopulations are delineated, the assessor then tests for ESUs in Phase 2. The scores from Phase 1 do not carry over to Phase 2, because in Phase 2 new candidate scenarios can be tested as it is unlikely that the number of subpopulations and ESUs are identical. A minimum score of 10 is needed to delineate distinct ESUs. This can only be reached if genetic or genomic evidence is collected for the species (Figure 2B and Table 1). At least one genetic evidence category is needed as a 'yes', coupled with either another genetic evidence category or with significant differences in inherited or acquired characteristics and/or traditional knowledge, to support a scenario with distinct ESUs. If less than 10 points are scored, the candidate scenario can either be regarded as (a) 'not supported', because 10 points cannot be reached or exceeded even if all missing data were supportive of the tested scenario; or (b) as a 'possible ESU (pESU)' scenario, because the minimum threshold of 10 points could be reached or exceeded if missing data subsequently prove to be supportive of the tested scenario. However, if a candidate scenario currently only scores in recorded biological evidence-types (so non-genetic), the scenario should be regarded as 'data-deficient' rather than as a pESU scenario. At the end of Phase 2, assessors should select the scenario with the highest score.

If no candidate scenarios can reach the minimum threshold of two points in Phase 1 or exceed 10 points in Phase 2 (even if missing data could be acquired) the species may comprise a single subpopulation and therefore a single ESU. Assessors can specifically test a scenario with one subpopulation (Phase 1) and one ESU (Phase 2). However, for this scenario only points have to be given if there is compelling evidence for 'no' distinctiveness of the tested lines of evidence within the species (criteria are outlined in Table S1 in the supplementary material, section A). The same scoring system as for testing multi-subpopulations or -ESU scenarios applies.

This framework has already been tested thoroughly on variety of species across different taxonomic groups (details in the supplementary material, Section B, Table S2) with two detailed examples shown (Figure 3). The thresholds of 2 points in Phase 1 and 10 points in Phase 2 are based on the extensive testing with species expressing different data availabilities. We envision that as the framework is applied, more evidence-types and exceptions will arise, and the framework could be adjusted accordingly. To facilitate easier application and scoring we aim in the future to develop a web platform for assessments. In order to test this framework and further improve it, we aim to test species listed in the IUCN Green Status of Species framework.

ESU and subpopulation division - risks and limitations

Choosing whether or not to split species into smaller units comes with an inherent risk of 'under- or over-splitting' that is amplified when non-molecular data are used because genetic divisions may be cryptic (Frankham et al., 2019). Over-splitting a species into several ESUs for IUCN assessment could artificially inflate estimated extinction risks. Conversely, under-splitting a species may give a falsely optimistic picture of extinction risk or Green Status that inhibits species management actions, in turn exacerbating extinction risk and fuelling genetic diversity loss (Frankham et al., 2019; Liddell et al., 2021). An extensive risk assessment has been developed for the framework to help assessors balance these challenges (supplementary material section C). However, balancing the patterns identified with their uncertainty requires genetic knowledge. Authors conducting molecular research can support managers and assessors by offering clear lay summaries of their results in their publications and depositing their data in open access repositories to support reuse (Leigh et al., 2024). In turn assessors can seek advice from trusted sources during evaluations.

Though we have developed our framework specifically to support IUCN assessments, we acknowledge dividing species into subpopulations or ESUs could unintentionally impact downstream conservation management decisions. A recent meta-analysis showed there is a strong historical tendency in conservation management to define units as genetically distinct through data misinterpretation and/or weak or no evidence (Liddell et al., 2021). These divisions have previously led to erroneously isolated units that unnecessarily increased extinction risk (e.g., *Perameles gunni*; Weeks & Rypalski, 2021). Based on the history of genetic unit mismanagement, we strongly stress that division of species into subpopulations or ESUs does not necessarily indicate a need to manage units in isolation (e.g., Senn et al., 2014). Identifying within-species units can help conservation managers by supporting ex-situ management or conservation translocation evaluation. They also support targeted actions designed to restore connectivity e.g., by identifying appropriate source subpopulations or ESUs, and generating evidence to consider when deciding whether mixing or separate management is the best way to achieve a conservation goal (Liddell et al., 2021).

In the future, advances in genomics will provide us with deeper biological insights that could be relevant to management. For example, genetic load consists of deleterious alleles whose frequency and presence can vary across isolated genetic units. Undoubtedly, genomic diversity could become increasingly important for the effective management of ESUs (Dussex et al., 2023). Nevertheless, we have not yet included genetic load as a criterion in this framework, because it remains challenging to identify and is less relevant for ESUs delineation, which focuses on the evolutionary heritage that conservation managers aim to preserve (van Oosterhout et al., 2025).

How Evolutionarily Significant Units could fit into existing IUCN Frameworks?

The framework proposed here is intended to support the integration of subpopulations and ESUs in existing IUCN frameworks in a standardized fashion (Red List and Green Status of Species). We note that the inclusion of ESUs will not impact the stability of the Red List nor impede temporal comparisons because it represents a new listing level that could receive its own status separate from the species status as a whole, subspecies or subpopulation status (IUCN, 2024a). Within the IUCN Red List, these within-species units could be listed under the taxonomy section and could help to ensure a standardized way to reference and acknowledge

the value of within-species genetic diversity. Separate extinction risk assessments of ESUs could add value beyond existing Regional, National and subspecies Red Listings. For the IUCN Green Status of Species framework (IUCN, 2021), ensuring retention of historical genetic diversity, in addition to the current goals of recovery of historical species range, could help to aid long-term species recovery. Once subpopulations or ESUs are delineated, genetic data or proxies could be incorporated into the Green Status of Species assessment through the ecosystem functionality score (IUCN, 2024b).

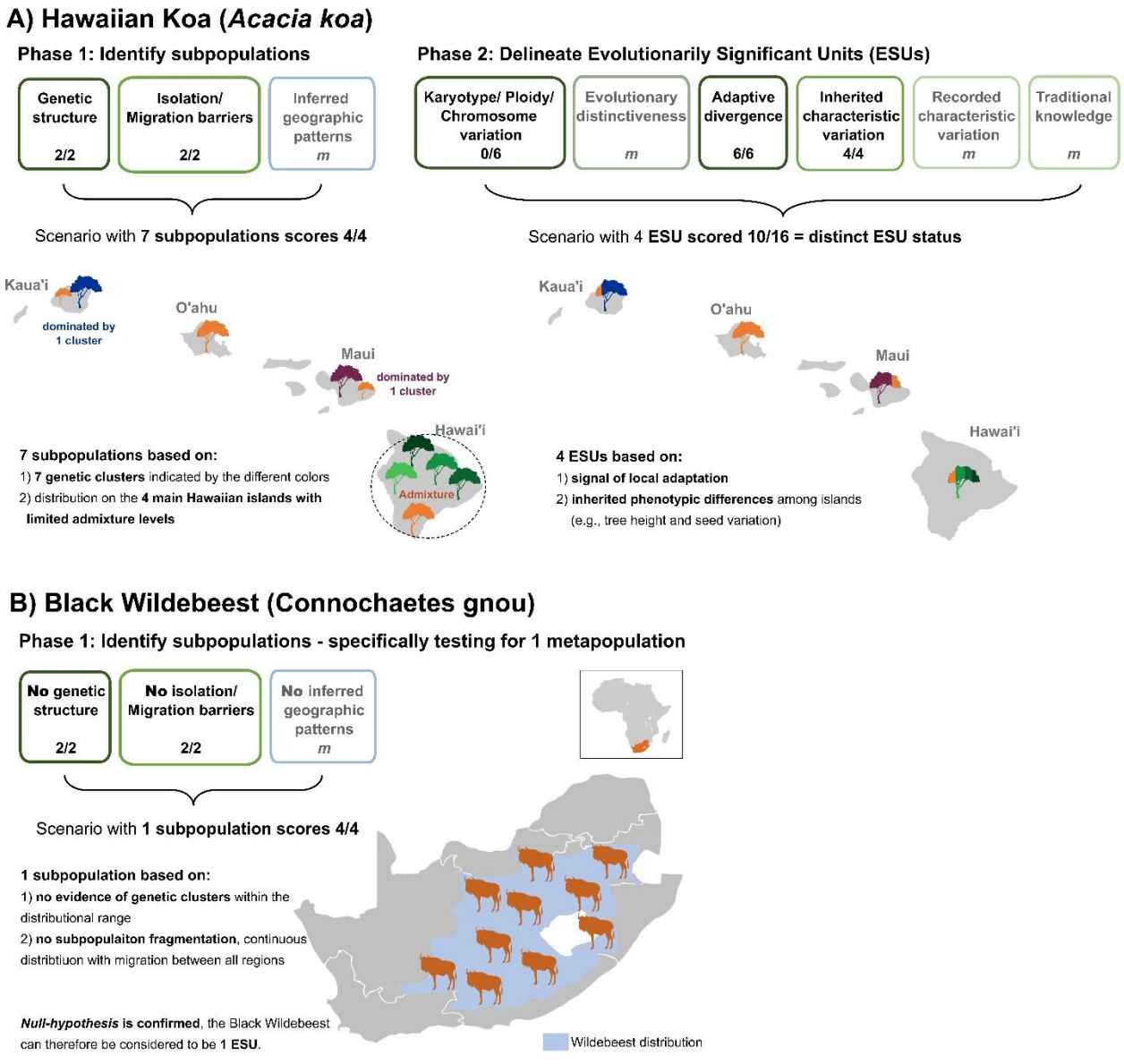

Figure 3 - Visual summary of a case study assessment of A) the Hawaiian Koa tree (Acacia koa) and B) the Black Wildebeest (Connochaetes gnou). The case studies contain a summary of the lines of evidence with missing lines of evidence shown in grey as they were considered as data-deficient. Scores for each scenario are as a proportion of total possible score (e.g., Phase 1 score 4 points out of 4 possible points tested because there is no data for Inferred geographic patterns). To support visualisation the lower panel contains a pictorial description of the divisions in the best supported scenarios. More details are given in the supplementary material, Section B.

Both phases of this framework also support Red List assessments under Criterion B: species with severely fragmented subpopulations, where individuals are found in small and isolated units, and thus have an increased risk of extinction (IUCN, 2024a). Severe fragmentation is currently inferred from habitat fragmentation alone, but subpopulations belonging to the same ESU showing high human-mediated genetic differentiation could be used as an additional form of evidence. Furthermore, identification of ESUs could help determine whether subpopulation fragmentation is recent and harmful, or ancient and unlikely to impact extinction risk.

Delineation of ESUs can also assist with prioritizing site-based protection measures. Many conservation measures are focused around site-based protection (e.g., 30x30, Protected and Conserved Areas; (CBD, 2022). Understanding which ESUs are present could help highlight the differential conservation value of different sites across a species' range and ensure that unique ESUs are not overlooked and lost. The IUCN tool that has been recently developed to assist with the prioritization of sites for protection is the 'Global Standard for the Identification of Key Biodiversity Areas' (KBAs; IUCN, 2016). The KBA standard uses a species-centric approach. Although ESUs are generally likely to span multiple KBAs, a standardized ESU delineation may be helpful in adding additional evidence for appropriate delineation of KBAs under the criteria (vi) 'distinct genetic diversity' measure of criteria A1, B1 & B2 (Threatened species, Individual and co-occurring geographically restricted species). The standardized ESU delineation may also support relative value assessments of KBAs for species that are not threatened or range-restricted (i.e., to be delineated under criteria D&E).

In this paper we outlined a two-step framework to standardize and delineate subpopulations and ESUs by integrating genetic and non-genetic (recorded biological and inferred) evidence. Implementation of this framework could support incorporation of unique and meaningful genetic units in species assessments. This information is critical to inform conservation priorities and assist in slowing global genetic diversity decline.

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Author contribution statement

This manuscript and project were led by JCG and DML.
The funding for the IUCN workshop was secured by SP-E and GS.
MEH, IMR, GS, SP-E, JG, DML acted as the project steering committee.
EJ secured funding for a follow up workshop through Newcastle University.
LDB, PB, AB-O, JMdS, JAD, AF, JAG, CEG, MEH, CH, AK, AJM, ELJ, AJP, JCP, IMR, HS, GS, PS, CVO all contributed to the discussions, conceptualization, and writing of the framework and manuscript.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Supplementary material: Practical genetic diversity protection: an accessible framework for IUCN subpopulation and Evolutionarily Significant Unit identification

Content

A) Scoring scheme for testing 1 subpopulation and 1 ESU - details and examples

This section is to provide more details and examples to the scoring system of the two-step framework for subpopulation and ESU delineation. The developed framework is standardized for scenarios with several subpopulations and ESUs. However, in case assessors only want to test 1 subpopulation in Phase 1 as the null hypothesis or only 1 ESU in Phase 2, the way information is scored (points given) have to be adjusted (see Table S1).

Table S 1 - Adjustments to the lines of evidence used in the two-step framework to delineate subpopulations and ESUs, when only testing for 1 subpopulation (in Phase1) or 1 ESU (Phase 2).

Types of evidence	Description	Example methods of assessments of 1 subpopulation/ESU	Example studies using lines of evidence	Score
Genetic evidence				
Genetic structure *(Phase 1)*	To provide support for the presence of only one subpopulation, there should be compelling evidence for absence of genetic structure.	Structure-like analyses, differentiation statistics (pairwise F_{ST} values), private alleles within subpopulations.	e.g., Abbott & Double, 2003	**No** genetic structure = 2
Karyotype, ploidy, and chromosome structure variation *(Phase 2)*	To provide support for the presence of only one ESU, chromosome variation can be present within a species but should not be coupled with consistent spatial variation or unfit hybrids.	Evidence of heritable differences from cytogenetic analysis and structural variant detection software coupled with evidence of no/rare/unfit hybrids between proposed units.	e.g., Ahrens et al., 2020; Ferreira et al., 2017; Hollenbeck et al., 2022	**No** variation = 6

Evolutionary distinctiveness *(Phase 2)*	To provide support for the presence of only one ESU, the species should be a well-connected metapopulation with no signs of restricted gene flow.	Long evolutionary divergence times and/or reciprocal monophyly. In complex cases follow statistical support (above 75% support on branches that define a clade), derived from a statistically valid evolutionary model and tree-building approach.	e.g., Moritz, 1994; Moritz & Faith, 1998; Walsh et al., 2024	**No** evolutionary distinctiveness = 6
Adaptive divergence *(Phase 2)*	To provide support for the presence of only one ESU, there should be evidence confirming there is no local adaptation across different parts of the species range.	Robust genomic signals of local adaptations or stable hybrid zones.	e.g., Bonin et al., 2007; Rodríguez-Quilón et al., 2016	**No** adaptive divergence = 6

Recorded biological evidence

Isolation and barriers to migration *(Phase 1)*	To provide support for the presence of only one subpopulation, there should be evidence of a continuous occupation of the range with frequent migration between all regions.	Occupation of different biogeographical zones. Occupation of discrete remnants of historical habitats with little to no chance of natural migration between habitat patches.	e.g., Hewitt, 2004; Lorenzen et al., 2012	**No** isolation and barriers = 2
Inherited characteristic variation *(Phase 2)*	To provide support for the presence of only one ESU, there should be evidence that there are no heritable differences in important traits across the species range.	Heritable differences in focal traits observed in common garden experiments or other robust analytical tests (e.g., cross fostering).	e.g., Small et al., 1998; Wainwright et al., 2008	**No** inherited characteristics = 4

Recorded characteristic variation *(Phase 2)*	To provide support for the presence of only one ESU, there should be evidence of no differences in traits across the species range.	Traits differ between units but may be acquired or transmitted (non-genetic). For example, cultural and/or learnt behavioural differences like foraging techniques, alternative migration routes, regional birdsong 'dialects'; methylation differences; body size differences.	e.g., Lundberg et al., 2017; Sanchez-Donoso et al., 2022; Toews et al., 2019	**No** recorded characteristics = 2
Traditional knowledge *(Phase 2)*	To provide support for the presence of only one ESU, there should be knowledge that all individuals across the range are similar and freely interbreed or move.	Recorded information that stems from indigenous or local knowledge (according to the IUCN ILK framework, IUCN 2022). This encompasses information that is not yet statistically analysed, information that is analysed should be counted as inherited or recorded characteristics.		**No** differences according to traditional knowledge = 2
Inferred evidence				
Inferred geographic patterns *(Phase 1)*	To provide support for the presence of only one subpopulation, models should show evidence that there is no differentiation or fragmentation.	Subpopulation disjunction, phylogeographic and/or biogeographic evidence from co-occurring species used as a proxy, assumption of biogeographical data/refugia without direct evidence, dispersal distances and buffer (also used in the CBD genetic indicators, Hoban et al., 2020)		**No** inferred geographic patterns = 1

B) Detailed information on Case studies

1) List of species tested with the proposed framework

Table S 2 – List of species tested with the proposed framework. Each of those species was tested thoroughly with different candidate scenarios. The high scoring scenarios are now described here and points given for Phase 1 and 2 are indicated. Points are displayed the following scored points/evaluated points (= lines of evidence with available data).

Species	Phase 1	Phase 2	Data available
Common Eland (*Tragelaphus oryx*)	**2 subpopulations** (4/4 points)	**1 ESU** (12/18 points)	Mitochondrial DNA
Cape Mountain Zebra (*Equus zebra*)	**2 subpopulations** (4/4 points)	**2 possible ESUs** (8/14 points)	Mitochondrial and microsatellite data
Cape buffalo (*Syncerus caffer*)	**2 subpopulations** (4/4 points)	**2 ESUs** (22/22 points)	Nuclear genomes, mitochondrial DNA
Rodriques fruit bat (*Pteropus rodricensis*)	**1 subpopulation** (4/4 points)	**1 ESU** (Null-hypothesis confirmed)	Microsatellite data
Iberian lynx (*Lynx pardinus*)	**2 subpopulations** (2/4 points)	**1 ESU** (10/16 points = human mediated admixture due to captive breeding and translocations)	Microsatellite and WGS (whole-genome sequencing) data
African penguin (*Spheniscus demersus*)	**1 subpopulation** (2/4 points)	**1 ESU** (Null-hypothesis confirmed)	Microsatellite data
Western leopard toad (*Sclerophrys pantherine*)	**4 subpopulations** (2/4 points)	**2 possible ESUs** (6/12 points)	Mitochondrial and microsatellite data
Yellow-tufted honeyeater (*Lichenostomus melanops*)	**2 subpopulations** (4/4 points)	**2 possible ESUs** (8/14 points)	Microsatellite data
Sable Antelope (*Hippotragus niger*)	**5 subpopulations** (4/4 points)	**5 possible ESUs** (6/16 points)	Mitochondrial genomes
Black rhinoceros (*Diceros bicornis*)	**7 subpopulations** (4/4 points)	**7 possible ESUs** (6/16 points)	Mitochondrial DNA, microsatellites, nuclear genomes
Rewarewa tree (*Knightia excelsa*)	**2 or 4 subpopulations** (2 scenarios scored 4/4 points = Assessment should be done by species experts)	**2 ESUs** (12/16 points)	WGS (whole-genome sequencing) data
Hawaiian Koa (*Acacia koa*)	**7 subpopulations** (4/4 points)	4 ESUs (10/16 points)	Microsatellite and GBS (genotyping-by-sequencing) data
Black Wildebeest (*Connochaetes gnou*)	**1 subpopulation** (4/4 points)	**1 ESU** (Null-hypothesis confirmed)	Mitochondrial and microsatellite data

2) Hawaiian Koa (*Acacia koa*)

Background

The Hawaiian Koa is an endemic tree species of high ecological (e.g., provides key habitat for many threatened species and watershed recharge areas), economical (e.g. highly regarded hardwood timber) and cultural importance (e.g., timber is used to build traditional Hawaiian outrigger canoes for fishing, racing, and voyaging) (Baker et al., 2009; Mitchell et al., 2005). It is distributed on the four main Hawaiian Islands (Hawai‘i, Maui, O‘ahu and Kaua‘i). Hawaiian Koa grows in various habitats ranging from wet to drier areas in different elevations and therefore also expresses high variation in phenotypic traits (Baker et al., 2009). It is currently listed under the threat category ‘least concern’ in the IUCN Red list; however the last assessment of Koa was in 2010 (Contu, 2010). In 2015 an action plan for Hawaiian Koa was developed to discuss the actions needed to achieve healthy koa forests that accommodate people and economic needs and promote forest conservation (Inman-Narahari, 2015).

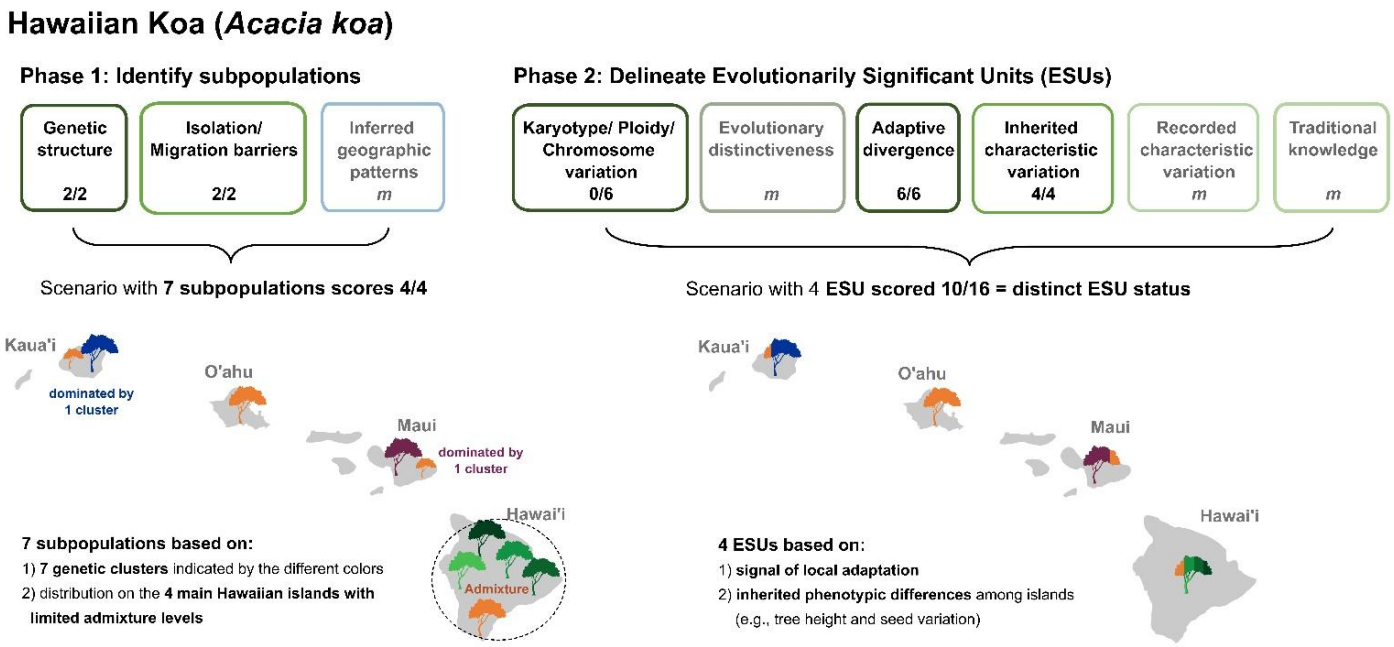

Figure S 1 - Framework schematic summary for the case study of the Hawaiian Koa (Acacia koa). In Phase 1 a scenario with 7 separate subpopulations scored high enough to move to Phase 2, where a scenario of 4 potential ESUs was tested. Lines of evidence categorized as missing (=data-deficient) are greyed out. Graphic is based on a freely available png of the Hawaiian Islands (cleanpng.com) and was adapted for this study.

Phase 1

We tested two different candidate scenarios: (1) two subpopulations based on genetic data (microsatellite markers; Fredua-Agyeman et al., 2008), (2) seven potential subpopulations, based on genomic data (genotyping-by-sequencing (GBS); Gugger et al., 2018) (Table S2).

Table S 3 - Scoring table for Phase 1 for the Hawaiian Koa (Acacia koa). Candidate scenarios tested are displayed in the columns and line of evidence scored in rows. The final score for each candidate scenario is displayed in the last row.

	Subpopulation - scenario 1 2 subpopulations (microsatellite dataset)	**Subpopulation - scenario 2** 7 subpopulations (genomic dataset)
Is there evidence of genetic structure? **2 points** *Evidence through (multiple can apply):* • *Population Clustering Analyses* • *Pairwise F_{ST} Values* • *Private Alleles*	☑ Yes ☐ No ☐ No data	☑ Yes ☐ No ☐ No data
Are there records of non-human mediated isolation or subpopulation fragmentation (through migration barriers)? **2 points** *Evidence through (multiple can apply):* • *Occupation of different biogeographical zones* • *Occupation of discrete remnants of historical habitats with little/no natural migration*	☐ Yes ☑ No ☐ No data	☑ Yes ☐ No ☐ No data
Is there indirect evidence of likely subpopulation fragmentation? **1 point** *Evidence through (multiple can apply):* • *Subpopulation disjunction* • *Phylogeographic and/or biogeographic evidence from co-occurring species* • *Assumption of biogeographical data/refugia without direct evidence* • *Dispersal distance and buffer according to the CBD indicators*	☐ Yes ☐ No ☑ No data	☐ Yes ☐ No ☑ No data
Final scores	**2/4**	**4/4**

For the three different lines of evidence within Phase 1, the candidate scenarios one (2 subpopulations) and two (7 subpopulations) scored 2/4 and 4/4 points, respectively. Both subpopulation - scenarios reached the minimum threshold of 2 points, however the second scenario scored higher (4/4). The high

score scenario determined 7 distinct subpopulations based on **genetic clustering analyses and pairwise *F*ST values.** Hence a score of 2/2 was given to the 'genetic structure' category. Subpopulations were **distributed on the various Hawaiian oceanic islands** (Hawai'i, Maui, O'ahu and Kaua'i) with only limited admixture levels between islands (suggestive of isolation with occasional dispersal) (Figure S1). However, the occurrence of the same genetic cluster among some of the islands was explained by recent 'human-mediated' dispersal, potentially as a side product of restoration programs by Gugger et al. (2018). Natural patterns of occurrence show a more distinct **occupation of different habitats which could be considered as different biogeographical zones and an indication for migration barriers** and led to the allocation of a score of 2/2 in this category. For the third line of evidence describing **indirect evidence of subpopulation fragmentation**, a score of 0/2 was given for both tested scenarios, since we could not find any evidence for this category and so it was considered missing.

Phase 2

We decided to only test 1 candidate scenario, comprising four potential ESUs for Phase 2 (Table S3). In the landscape genomics study of Gugger et al. (2018), it was shown that genetic differentiation is rather limited among the four main Hawaiian Islands, but admixture was detected between subpopulations on the Island of Hawai'i. Another study based on microsatellites suggested that each Hawaiian Island could constitute a separate unit/entity ('Candidate scenario 2' in Phase 1; Fredua-Agyeman et al., 2008). Based on the admixture pattern found on the Island of Hawaii (see Gugger et al., 2018) we grouped the extant five subpopulations on this Island and treated them as one ESU. Each of the other three islands are dominated by one genetic cluster, so those were regarded as three separate ESUs.

Table S 4 - Scoring table of Phase 2. The first column comprises the different lines of evidence in the form of a question with example data which can be used as 'evidence' and its score. The second column indicates if there is evidence of distinctiveness ('Yes') or not ('No'), or whether no data are available ('No data').

	ESU - Scenario 1 4 ESUs (each Island its own ESU)
Is there inherited variation in chromosome numbers, ploidy level or chromosome structure? **6 points** *Evidence through (multiple can apply):* *a. Karyotype variation* *b. Difference in ploidy levels between units* *c. Structural variation of chromosomes between units*	☐ Yes ☑ No ☐ No data

Is there evidence of long-term reproductive isolation? **6 points** *Evidence through (multiple can apply):* *a. Reciprocal monophyly* *b. Molecular Estimates of divergence time*	☐ Yes ☐ No ☑ No data
Is there evidence of local adaptation or adaptive divergence? **6 points** *Evidence through (multiple can apply):* *a. Genomic signals of local adaptation* *b. Evidence of stable hybrid zones*	☑ Yes ☐ No ☐ No data
Is there evidence of inherited characteristic differences? **4 points** *Evidence through (multiple can apply):* *a. Heritable differences in focal traits observed in experiments or confirmed by analytical tests*	☑ Yes ☐ No ☐ No data
Is there evidence supporting recorded characteristic differences? **2 points** *Evidence through (multiple can apply):* *a. Cultural or learnt behavioral differences unique or specific between units* *b. Acquired or transmitted traits (e.g., migration pattern, methylation differences, body size differences)*	☐ Yes ☐ No ☑ No data
Is there traditional knowledge suggesting distinctiveness between units? **2 points** *Evidence through (multiple can apply):* *a. Recorded differences based on traditional and local knowledge*	☐ Yes ☐ No ☑ No data
Final score	**10/16**

In the tested candidate scenario of 4 distinct ESUs, there was **no karyotype variation** detected among different samples collected from all four islands (all are tetraploid (2n=52), Shi, 2003). Therefore, a score of 0/6 was allocated in the 'Karyotype variation' category. Taxonomy and phylogeography of the Hawaiian Koa is still only disentangled to a limited extent (e.g., Baker et al., 2009) and so **no data on long-term reproductive isolation** are available, resulting in missing data in the 'Evolutionary distinctiveness' category. **Signals of local adaptation** were detected by conducting genome wide associations with environmental variables. Precipitation variables showed the strongest correlations to genetic divergence among islands and was explained to play an important role in dealing with water stress (Gugger et al., 2018). **Inherited phenotypic differences** based on provenance tests were

detected. General adaptations to the tree's home environment were detected in the form of differences in growth form and seed shape (e.g., Baker et al., 2009). Therefore, the categories 'Adaptive divergence' and 'Inherited characteristic variation' were awarded 6/6 and 4/4 points respectively. Evidence of **recorded characteristic variation is not available**, resulting in missing data in this category. This case study is based on literature, consequently there is a **lack of deep understanding of traditional knowledge** about the different Hawaiian koa subpopulations. For this reason, this category was also declared as missing.

The candidate ESU scenario tested here (7 subpopulations = **4 ESUs**) scored 10 points out of 16 points evaluated (with available data). The tested scenario reaches the threshold to gain **distinct ESUs status** by scoring points in one genetic line of evidence ('Evolutionary distinctiveness') coupled with one recorded line of evidence ('Inherited characteristic variation') leading to a designation of 6/6 and 4/4 points. Three lines of evidence ('Evolutionary distinctiveness', 'Recorded characteristic variation' and 'Traditional knowledge') are missing for the Hawaiian Koa tree, leading to a designation of 0/6, 0/2 and 0/2 points, respectively.

3) Black Wildebeest (*Connochaetes gnou)*

Background

Black wildebeest (*Connochaetes gnou)* or the white-tailed gnu is one of the two closely related wildebeest species, the other species being the blue wildebeest (*Connochaetes taurinus*; Grobler et al., 2018). Natural populations of *Connochaetes gnou* are endemic to southern Africa with a historical range including South Africa, Eswatini and Lesotho (Vrahimis et al., 2016). The species has been hunted to extinction in Eswatini and Lesotho during the 19th Century but has now been reintroduced to Eswatini/Lesotho and more recently introduced to Namibia where populations have become well established. Black wildebeest inhabits different habitats such as grasslands, open plains and karoo shrublands. Current threats to the species may include the potential hybridisation with the closely related blue wildebeest where in close proximity (Grobler et al., 2011; Vrahimis et al., 2017). After the 19th Century, fewer than 300 individuals remained (Estes et al., 2004; Von Richter, 1972). The species was rescued from these individuals and currently there are about 16,000 individuals with 7,000 individuals in Namibia (outside their natural range; Vrahimis et al., 2016). About 20% of black wildebeest is confined in protected areas while the majority (80%) of individuals occur in privately owned land. Population growth is now trending upward and for this reason the IUCN rates the species as being of least concern (Lundrigan & Bidlingmeyer, 2000; Vrahimis et al., 2017).

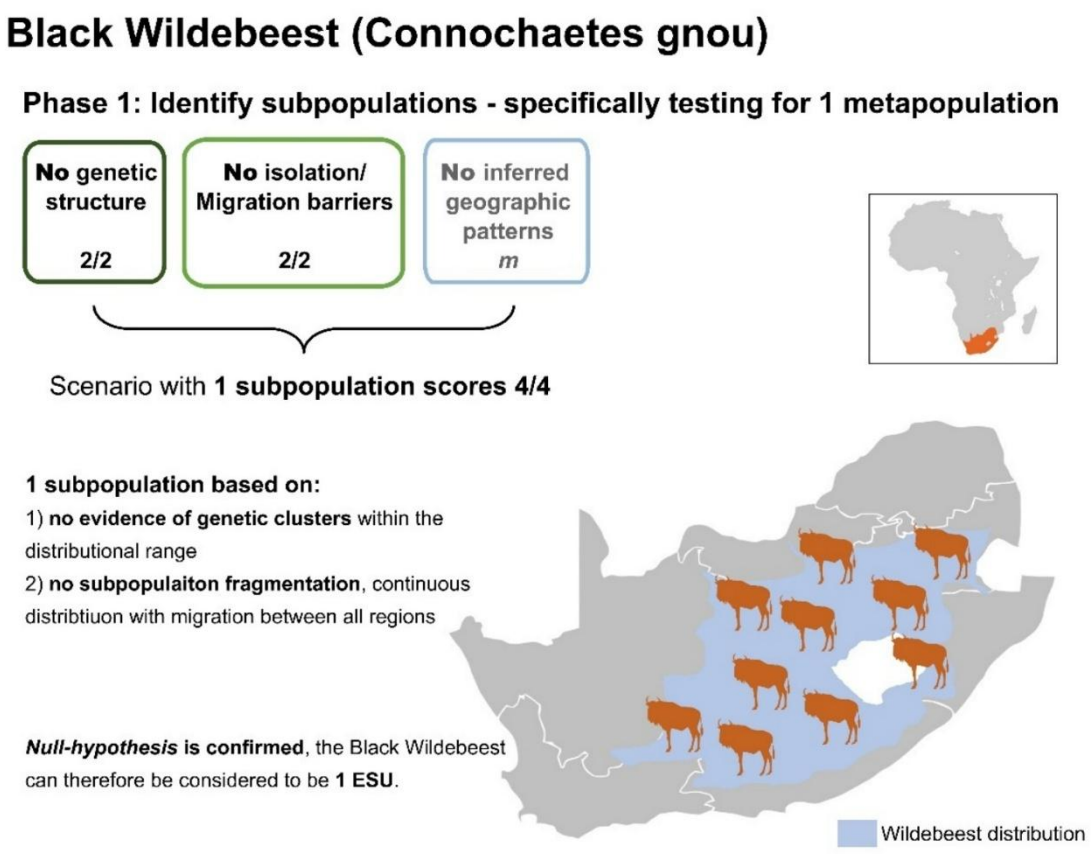

Figure S 2 - Framework schematic summary for the case study of the Black wildebeest (Connochaetes gnou). Based on previous knowledge only one scenario in Phase 1 was tested (1 subpopulation). Lines of evidence categorized as missing (=data-deficient) are greyed out. Graphic is based on a freely available png of South Africa (cleanpng.com) and was adapted for this study.

Phase 1

This is an example where we tested for only one subpopulation based on genetic data (mitochondrial DNA, microsatellite markers; Corbet & Robinson, 1991; Grobler et al., 2018; Table S4). Here we are testing whether the entirety of the species represents one subpopulation, in other words, there should be no evidence of genetic structure, human mediated isolation/fragmentation or indirect subpopulation fragmentation.

Table S 5 - Scoring table for Phase 1 for the black wildebeest. The questions are based on Table S1, since only one candidate scenario based on one subpopulation is tested here. The final score for the tested candidate scenario is displayed in the last row.

	Subpopulation - scenario 1 1 subpopulation
Is there evidence for an absence of genetic structure? **2 points** *Evidence through (multiple can apply):* • *Population Clustering Analyses* • *Pairwise Fst Values* • *Private Alleles*	☑ Yes ☐ No ☐ No data
Are there records of continuous occupation of the range with frequent migration between all regions? **2 points** *Evidence through (multiple can apply):* • *Occupation of different biogeographical zones* • *Occupation of discrete remnants of historical habitats with little/no natural migration*	☑ Yes ☐ No ☐ No data
Is there indirect evidence of no subpopulation fragmentation? **1 point** *Evidence through (multiple can apply):* • *Subpopulation disjunction* • *Phylogeographic and/or biogeographic evidence from co-occurring species* • *Assumption of biogeographical data/refugia without direct evidence* • *Dispersal distance and buffer according to the CBD indicators*	☐ Yes ☐ No ☑ No data
Final scores	**4/4**

For the three different lines of evidence within Phase 1 (based on Table S1) only one candidate scenario was assessed (1 subpopulation). Based on mitochondrial DNA haplotypes and microsatellite markers **no evidence of genetic clustering** was detected. A score of **2/2** was given for this line of evidence. Clusters of subpopulations are **continuously distributed across the central region of South Africa** which extends into Eswatini and Lesotho **with migration between all regions** (Figure S2). Some human-mediated translocations between privately owned populations have been granted and these actions have not affected genetic diversity patterns. This led to the allocation of a score of **2/2** for the second line of evidence (**no subpopulation fragmentation)**. The third line of evidence scored **0/2** because no data are available to assess this line of evidence (**indirect evidence of no subpopulation fragmentation**). Therefore, the total score for this scenario was 4. For the three different lines of evidence within Phase 1, the scenario of one subpopulation scored **4/4**. Therefore the 1 subpopulation scenario scored 4 points (and

a minimum of **2 points is needed)**, so the **null-hypothesis can be accepted** and the black wildebeest can be considered to represent one subpopulation/metapopulation and therefore also as one Evolutionarily Significant Unit (ESU).

C) Risk Assessment

a) What does the risk assessment section do?

The risk assessment can be used in conjunction with the Evolutionarily Significant Unit (ESU) delineation framework. It highlights the risk of misinterpretation of the available data and therefore assigning a wrong score for a given line of evidence. Specifically, it goes through each line of evidence and discusses the risk of over-splitting and under-splitting species into ESUs. This risk assessment should act as a general guide and is not exhaustive, contacting original authors or using expert interpretation may be needed, particularly for complex scenarios.

General risk will be present throughout the framework due to different data resolutions and study power. We stress that interpreting data requires appreciation and consideration of the difference between biological and statistical significance of the data in question:

Statistical significance in genetic estimators (e.g., a p-value), reflects the degree of certainty with which a parameter may be distinguished from a null hypothesis (typically "zero effect", e.g., no genetic differentiation) (Nakagawa & Cuthill, 2007; Sokal & Rohlf, 2009).

Biological significance refers to the ecological or evolutionary magnitude of genetic measurements, such as the degree of genetic differentiation.

Importantly, statistical significance is determined by considering the magnitude of an effect alongside its precision/error, so statistical significance is often related to biological significance, but not always. Statistical significance in genomics does not always equate to biological significance because small effect sizes may become statistically significant in "big data" genomics analysis (Lo et al., 2015). Distinguishing between the two requires subject-matter expertise to interpret whether a particular difference in measurement is biologically meaningful and to what degree. For example, a large biological effect estimated with poor precision (e.g., because few molecular markers or samples were analysed), might not be statistically significant. Caution interpreting the measurement is warranted, because poor precision and statistical non-significance suggest difficulty in differentiating the result from zero, whereas the large magnitude of the result (biological significance) may justify further investigation and a more thorough sampling strategy. Conversely, a measurement that is small but very precise (e.g., because a very large number of genome-wide markers or samples were used) may be statistically significant, and thus we can be fairly confident the estimate is not zero, but if the magnitude

of the measurement is small, it may not be biologically meaningful and thus not be relevant for the designation of conservation units.

b) <u>What the risk assessment section does not do, and what other aspects of risk might need to be considered</u>

1. The risk assessment section does not evaluate the ecological and evolutionary "risks" or consequences of over-splitting and under-splitting *per se* for conservation management.
2. The risk assessment section cannot evaluate the "social" risks of changes in ESU designation. ESU designation could have (or be perceived to have) cultural or political meaning for stakeholders. Note that the IUCN Red List can be used to record indigenous names for species and indigenous taxonomies (https://www.iucnredlist.org/resources/ilk); this information may not always be complete. Further guidance on stakeholder inclusion can be sought from specialists, for example through the IUCN CEESP-SSC Sustainable Use and Livelihoods Specialist Group (https://www.iucnsuli.org/) or the IUCN-SSC Human-Wildlife Conflict and Coexistence Specialist Group (https://www.hwctf.org/).

<u>c) The Risk assessment for each line of evidence in Phase 1</u>

1. <u>Genetic structure</u>

Over-splitting

1) a STRUCTURE group or PCA cluster was used in isolation to identify a subpopulation (e.g., using softwares such as STRUCTURE; Pritchard et al., 2000 or PLINK; Purcell et al., 2007). Structure detection is dependent on subpopulation census size, isolation time, and sampling design. Detection of separate clusters can be strongly affected by sampling, e.g., sampling of individuals from a family group may drive a cluster that does not represent meaningful overall structure (Lawson et al., 2018; Liddell et al., 2021). Similarly, unbalanced sampling schemes (e.g., uneven number of individuals per subpopulation or patchy sampling of subpopulations within an isolation-by-distance pattern) can force discrete clusters to emerge that are in fact part of a continuum (Bradbury et al., 2018; Frantz et al., 2009; Perez et al., 2018). Notably small, isolated, or ex situ managed populations can rapidly become highly distinct due to random genetic drift, whereas reduced gene flow may take hundreds of generations to accrue when effective population sizes are large (Landguth et al., 2010).

2) Over-estimating differentiation by visual inspection of data rather than application of a statistical test. For example, STRUCTURE does not provide statistical support for differences among clusters, just for the number of clusters. Similarly, mtDNA networks are often inspected visually rather than applying a

test e.g., whether phiST (frequency plus sequence differences) is statistically significant between a pair (e.g., Garrick et al., 2004), or whether the network is within a 95% confidence interval (statistical parsimony) and 95% connection limit.

Risks for points 1& 2 can be mitigated by applying equal sampling, avoiding including relatives, applying multiple approaches and using statistical tests for evaluating model fit.

3) Over-interpreting private alleles. Private alleles are predominantly rare, hence subject to small sample sizes. To mitigate the risk of over-interpreting these data it is important to carefully consider what they are private relative to: e.g., a subpopulation that founded many other subpopulations may accordingly have shared alleles that would otherwise have been regarded as private, falsely reducing its apparent uniqueness.

Under-splitting

In general, the risk of under-splitting using genotypic population genetic data is considered to be low because the methods are highly sensitive to structure. Instances where under-splitting could be a risk include species with large effective population sizes that tend to possess low F_{ST} when analysed using microsatellite markers (Hedrick, 1999). Analysing genetic differentiation in such populations using a large panel of SNPs mitigates this problem. With such genomic data, even a small number of individuals can be used to detect significant genetic differentiation (Willing et al., 2012). Further mitigation includes understanding of site history and human-mediated change.

2. Isolation and barriers to migration

Over- or Under-splitting

1) due to misestimation of the level of lineage divergence and gene flow restriction assumed from the biogeographical information.
2) misinterpretation of human-driven habitat/environment segregation as a natural long-term pattern.
3) poor records or map-making.
4) species misidentification in the past.
5) misidentified environmental drivers.

Mitigation includes careful interrogation of the quality of available species- and distribution data.

3. Inferred geographic patterns

Over-splitting

1) Natural landscape/habitat differences can lead to separation of units without their being genetically distinct or different. Geographic distances/barriers can be caused by human interaction or interference (e.g., urban development, road construction, long-standing persecution etc). Co-occurring species used as proxies could, in fact, have very different evolutionary histories (e.g., Beavis et al., 2011; Stern, 2013).

2) Risk of over-splitting due to misestimation of the level of lineage divergence and gene flow restriction assumed from proxy/geographic/environmental/biogeographic information. Mitigation involves the use of genetic data on the target species (i.e see other lines of evidence).

Under-splitting

Speciation might not be detected if genetic data are absent. Co-occurring species used as proxies could, in fact, have very different evolutionary histories. Mitigation involves the use of genetic data on the target species (i.e. see other lines of evidence).

d) The Risk assessment for each line of evidence in Phase 2

1. Karyotype and chromosomal structural variation

Over-splitting

A risk of over-splitting arises by interpreting biologically inconsequential karyotype variation to support ESU status. Karyotypic variation can occur naturally within ESUs (e.g., Ahrens et al., 2020; Dobigny et al., 2017). Mitigation of the risk includes 1) identifying which forms of karyotypic variation are likely to have fitness consequences (e.g., ploidy differences, monobrachial homology), and 2) assessing evidence for fitness costs of karyotype mixing.

Under-splitting

A risk of under-splitting arises by 1) failing to detect consequential karyotypic variation due to insufficient sampling (sites or number of individuals) or insufficient technical resolution.
Note that karyotype studies remain somewhat uncommon and many species may be data-deficient and thus may not be successfully scored at this level.

2. Evolutionary distinctiveness

Over-splitting

A risk of over-splitting arises from:

1) over-interpreting reciprocal monophyly: it does not always reflect biologically meaningful divergence, because it can be driven by random genetic drift arising from small census size or demographic bottlenecks, as well as the statistical power of the molecular markers used (Kizirian & Donnelly, 2004; Rosenberg, 2003). Sole use of organelle-derived markers (e.g., mitochondrial haplotypes) is particularly high-risk, which can be mitigated by using them in conjunction with nuclear markers (Ballard & Whitlock, 2004; Zink & Barrowclough, 2008). For organelle-only data, keep the risk in mind and check other lines of evidence for incongruence with the groupings found here and if they arise, discuss the risks of over- or under-splitting with experts.

2) Over-interpreting long divergence times as support for the presence of ESUs. Apart from extremes e.g., millions of years vs. hundreds of years, divergence time thresholds do not clearly indicate the presence of an ESU. Recent splits between genetic units (<500 years) are likely to reflect human-mediated impacts on connectivity rather than natural eco-evolutionary processes. More ancient divergence times do not necessarily indicate the presence of ESUs. Mitigation of this risk should include interpreting data based on the history of the species and the known eco-evolutionary and human-mediated processes affecting putative ESUs (Jimoh et al., 2013).

3) Assuming that a lack of contemporary gene flow indicates a historical lack of gene flow (e.g., Roberts et al., 2011). Mitigation of this risk includes considering estimates of gene flow in relation to the timescale (i.e., number of generations) they pertain to, and assessing likely drivers of restricted gene flow.

Under-splitting

A risk of under-splitting ESUs arises from:

1) over-interpretation of lack of reciprocal monophyly. Two ESUs can have incomplete monophyly due to mtDNA introgression, incomplete lineage-sorting of nuclear genes, hybridization upon secondary contact, or more rapid evolution of selected traits than neutral background (Ballard & Whitlock, 2004; Edelman et al., 2019).

2) If variation in gene flow estimates from different parts of the genome are overlooked. Multiple neutral markers might indicate high levels of recent gene flow between two putative ESUs, while in contrast, regions of the genome underpinning major fitness characters might have highly restricted gene flow (e.g., in birds; Morales et al., 2018). Mitigation of these risks includes being mindful of inferential

power available from the marker types, marker density used, and sampling scheme of target sites (e.g., Edelman et al., 2019).

3. Adaptive divergence

Over-splitting

Risk of over-splitting occurs because:

1) genomic selection detection methods (e.g., F_{ST}-outlier programs) can easily show false signals of local adaptation (François et al., 2016). These can be very strong and show up in several tests. This is particularly true in units that have gone through a decline/bottleneck or in analyses that include even just a single bottlenecked site. Mitigation of these risks include using evidence from different outlier-detection methods (e.g., spatial, temporal), and using additional data about demographic history. Outliers from bottlenecked sites should not be considered sufficient evidence without corroboration from other lines of evidence.

2) Environment-based genomic selection detection methods can struggle to account for neutral genetic structure. Mitigation of these risks includes conducting robust studies with a reasonable number of focal sites, and replicated sampling sites across environmental designs.

3) Dividing into ESUs with hybrid zones can lead to over-splitting because these can be dynamic (i.e., move over time), and may be natural sources of gene flow. Mitigation of these risks includes temporal data on zone stability and treating the ESUs as co-dependent.

Under-splitting

A risk of under-splitting ESUs arises from:

1) failure to identify adaptation due to statistical power issues, and genome-wide assessment of potential candidates (Hoban et al., 2016). Thus the absence of outliers does not signal an absence of local adaptation and hence, when used in isolation, this line of evidence is not a compelling argument against units being distinct ESUs. This is particularly true for traits likely to be polygenic and in studies based on reduced-representation sequencing approaches providing only low density of markers across the genome (e.g, RADseq; Lowry et al., 2017). Mitigation includes ensuring adequate sampling across the genome and that multiple selection-detection methods are used.

Hybrid zones can be new or human-induced phenomena affecting historically distinct ESUs. Ignoring their historical context could potentially lead to under-splitting that may eventually lead to lineage replacement of an ESUs, or the formation of hybrid swarms.

4) Inherited variation in characteristics

Over-splitting

1) Risk of mistaking phenotypic plasticity for evolved adaptive phenotypic differences. Phenotypic plasticity i.e., when the same genotype produced different phenotypes in different environments, should not be included here.
2) Risk of overinterpreting phenotypic differences that are not biologically meaningful (e.g., rare colour morphs).
3) Risk of over-interpreting ecological traits of species (e.g., breeding time, use of spawning grounds) that are plastic and not inherited.
4) Risk of small/captive units showing a subsample of original range of phenotypic variation or phenotype being changed by inbreeding (i.e., "phenotypic drift" found in e.g., the Florida panthers; Johnson et al., 2010).

Mitigation includes adequate sampling so that between-unit variation can be understood in the content of within-unit variation, and understanding site demographic history and which phenotypic traits are likely to have strong environmental components. Conducting statistically sound experiments and tests to determine the inheritance of such traits.

Under-splitting

Not detecting evolved differences in phenotypic plasticity that would have been taken as evidence for different ESUs.

5) Recorded variation in characteristics

Over-splitting

1) Risk of mistakenly assuming acquired behavioural differences (e.g., learnt behaviour, migration patterns, foraging techniques) preclude interbreeding.
2) Risk of mistakenly assuming acquired phenotypic differences (e.g., methylation differences, body size differences) preclude interbreeding.

Can be mitigated by checking for assortative mating. If groups regularly interbreed, they are likely one ESU due to frequent gene flow.

Under-splitting

1) Likely to be data-deficient in many species, especially those that are hard to access or observe. Be aware of study selection bias.

2) Lack of data on behaviour may be due to lack of differences or an absence of attempted studies.

6) Traditional knowledge

Over- or Under-splitting

May occur if all relevant stakeholders are not consulted, or consensus cannot be reached among differing values-based positions. Mitigation is to conduct proper consultation and inclusion.

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