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Evolutionary Biology Centre

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From conservation genetics to conservation genomics using minimal-invasive sampling methods

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From conservation genetics to conservation genomics using minimal-invasive sampling methods

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1| Introduction: Species conservation in the Anthropocene

In the last centuries, anthropogenic pressures on wild animal populations have severely increased, causing extensive species and population losses (Pimm et al. 2014). Human impacts on the Earth's ecosystems are indeed so prominent that we can define our current time period as a new human-dominated geological epoch: the Anthropocene (Lewis & Maslin 2015). The Anthropocene is characterized by atmospheric CO2 levels not seen for at least the last 800,000 years and a high rate of species extinction. Thus, many consider the Anthropocene as the sixth mass extinction event (Barnosky *et al.* 2011; Ciais & Stocke 2013; Ceballos *et al.* 2015; Lewis & Maslin 2015). Although the magnitude of human-driven species extinctions is debated, even under a conservative scenario, current extinction rates are far above usual background levels (Figure 1) (Ceballos *et al*. 2015).

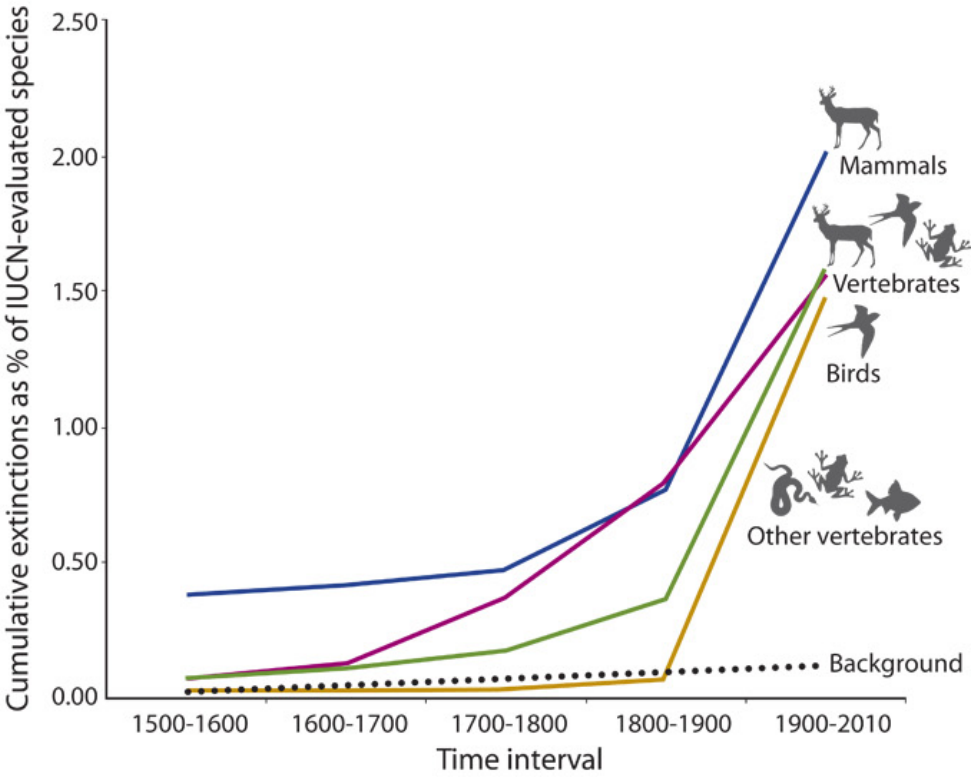

Figure 1. Cumulative vertebrate species recorded as extinct or extinct in the wild by the IUCN (conservative estimate). Graphs show the percentage of the number of species evaluated among mammals (5513; 100% of those described), birds (10,425; 100%), reptiles (4414; 44%), amphibians (6414; 88%), fishes (12,457; 38%), and all vertebrates combined (39,223; 59%). Dashed black line represents the number of extinctions expected under a constant standard background rate of 2 E/MSY. Figure obtained from (Ceballos *et al.* 2015).

The ongoing extinction rates result in the loss of approximately 58.000 species each year (using a conservation estimate of 5 to 9 million animal species on the planet), equal to 3 species every hour

(Scheffers *et al.* 2012; Costello *et al.* 2013). Decades ago, scientists already warned about the disastrous affects anthropogenic pressures have on wildlife and argued for the implementation of strict conservation plans (McVay 1973; Myers 1990). Historically, such species conservation plans have been primarily focussed on the prevention of extinctions, often by means of breeding programs or the extreme protection of small populations and individuals (Ferreira *et al.* 2012; Di Minin *et al.* 2015). Recently, attention has also been drawn to the impact of large-scale population declines rather than species extinctions itself, as small populations might not be viable in the long-term (Ceballos *et al*. 2017). Using a sample of 27.6000 terrestrial vertebrate species[*], Ceballos *et al*. (2017) showed that nearly half of the them experienced range and population declines within the last 100 year. All terrestrial vertebrate groups are affected, but mammals and amphibians suffer the most, with over 40% having experienced severe population decline (> 80%) within the last century (Figure 2).

The importance of biodiversity is supported by a wealth of literature: Biodiversity is vital for ecosystem functioning, provides ecosystem services and has a positive impact on human well-being *(Chapin III et al*. 1997; Hooper *et al*. 2005; Balvanera *et al*. 2006). Species extinctions and declining populations are thus not just undesirable *per se*, but also have direct impact on the quality of human lives. Without significant increase in conservation efforts, species extinction and declines are likely to continue at an alarming rate (Dawson *et al.* 2011; Costello *et al.* 2013; Costello 2015). In recent years, it has become widely recognised that biodiversity loss should be of major concern, and many countries have pledged towards increased worldwide conservation efforts. The fast majority of world governments is now commitment to halt human-induced extinctions and safeguarding important sites for biodiversity by 2020 under the United Nations Biodiversity Treaty (Balmford *et al.* 2005). Despite these commitments, we are far behind the 2020 targets. If we aim to meet the set goals, annual conservation funding has to increase by at least an order of magnitude (McCarthy et al. 2012).

[*] Obtaining reliable data for fish species is challenging due to their aquatic lifestyle. This group is therefore often excluded from estimates on species declines. However see (Ceballos *et al.* 2015).

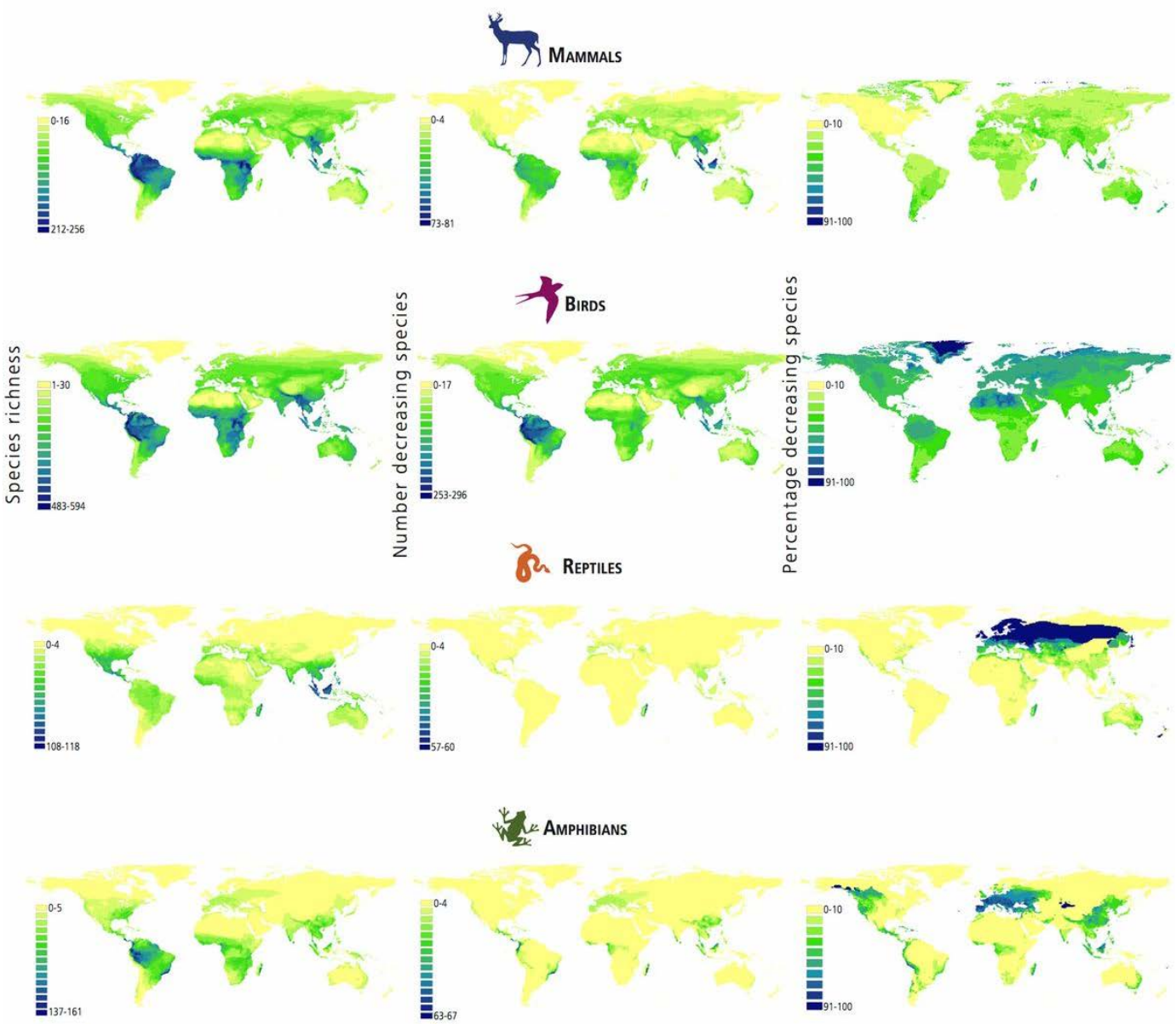

Figure 2. Global distribution of terrestrial vertebrate species according to International Union for Conservation of Nature, The IUCN Red List of Threatened Species, Version 2015.2 (IUCN, 2015). (Left) Global distribution of species richness as indicated by number of species in each 10,000-km2 quadrat. **(Center)** Absolute number of decreasing species per quadrat. **(Right)** Percentage of species that are suffering population losses in relation to total species richness per quadrat. The proportion of decreasing species per quadrat shows a strong high-latitude and Saharan Africa signal. However note that in absolute numbers of species experiencing population decline, regions of known high species richness are the most affected (Amazon, the central African region, and southeast Asia). Figure adjusted from (Ceballos et al. 2017)

Although this might seem unattainable in the near future, the significant reduction of extinction risks of all globally threatened species is estimated to cost around $76.1 billion annually, equal to just one tenth of the United States military budget (*The World Bank Indicators, https://data.worldbank.org*; McCarthy *et al.* 2012).

Alongside increased conservation investments, developments of novel and improvements of existing conservation methods to aid in the design of successful conservation strategies are urgently needed. One such promising development is the revolution in conservation genomics. Implementing genomic data into conservation is likely to become a prominent tool for the design of conservation strategies, as genetic data can be used to uncover relevant information for species conservation, such as population substructure, genetic connectivity and can be used to identify risks associated with demographic change and inbreeding (Frankham 1995). Additionally, populations with low genetic diversity are generally seen as having limited capacities to adapt to fast changing environments (Lande & Shannon 1996), display lower fertility (Reed & Frankham 2003), and are prone to infectious diseases (Smith *et al.* 2006). It is thus important to incorporate genetic diversity measures into efficient conservation planning. Multiple conservation success stories have relied on genetics (e.g. increase in Florida panther population size (Services 2008), detection of illegal harvesting and trade (Manel *et al.* 2002; Wasser *et al.* 2018), however despite its great potential, genomics is not yet widely used for conservation purposes. This is partly due to the fact that the most pressing conservation issues do not rely on genomics but rather require immediate political actions (Fyumagwa *et al.* 2013; Ripple *et al.* 2014). Additionally, translating genomic data into practical conservation programs is by no means straightforward (Shafer *et al.* 2015; Britt *et al.* 2018; Kardos & Shafer 2018). It is nonetheless widely agreed upon that genomics can play a crucial role in assisting long-term species conservation (Shafer *et al.* 2015).

In this essay I aim to discuss how the field of conservation genomics has developed in recent years and provide an outlook into future developments. I will specifically focus on genomic data obtained from

minimal-invasive sampling as such samples are the most prominent source of DNA in conservation oriented studies.

2.1| Obtaining genetic information from endangered species

An often overlooked challenge for the practical applications of conservation genomic tools is the possibility to obtain DNA samples for the animal species of interest. From the perspective of genomic analyses, high quality samples (e.g. fresh blood or tissue) are desirable. However, since most of the target species are extremely rare (frequently critically endangered), difficult to handle and especially vulnerable to human impacts, invasive sampling required to collect high quality DNA samples is rarely an option (Taberlet & Waits 1998). High quality genomic data from endangered animals is therefore often obtained from captive individuals. Although such samples can yield a wealth of information about the demographic history of a species (Locke *et al.* 2011; Scally *et al.* 2012), they often do not provide an accurate representation of the wild population. Specific breeding practices for captive populations can obscure demographic inferences due to changes in the social structure or "adaptations to captivity" of the individuals (Snyder *et al.* 1996; Araki *et al.* 2007; Christie *et al.* 2012). In rare cases, high quality genomic data has been collected from wild-born individuals of endangered species by means of invasive sampling. Such samples are often obtained by using biopsy darts, during medical treatment or post-mortem (Prado-Martinez *et al.* 2013; Xue *et al.* 2015; Foote *et al.* 2016; Abascal *et al.* 2016; Nater *et al.* 2017; Tunstall *et al.* 2018). Such studies mostly rely on opportunistic sampling, and therefore optimal sampling schemes cannot be designed and sample collection can take a long time (Fünfstück *et al.* 2015). For instance, the collection of high quality blood samples from seven critically endangered mountain gorillas individuals took over a decade (Xue *et al.* 2015). With the recent revolution in DNA sequencing technologies, sample availability rather than obtaining genomic sequence data is likely to be the main bottleneck (in terms of time and costs) for most conservation genomic projects. The limitations of access to high quality samples and ethical consideration in obtaining them were recognized already in the early days of conservation genetics

(Taberlet & Waits 1998). As means to overcome these impediments, much work has been done on optimizing the DNA yield and quality obtained from non-invasive sample sources. A clear definition of non-invasive sampling is lacking, but usually researchers mean that "*the animals are unaware of sampling and, therefore, are unaffected by it or animals are unrestrained and do not exhibit a chronic or severe stress response or experience reduction in survival or reproduction*" (Pauli *et al.* 2010). However, certain techniques can be unperceived or perceived by an animal, depending on the biology of the species and implementation of methodology and therefore I will use the more suitable term: *minimal-invasive sampling*. Minimal-invasive samples include among others faeces, hair, saliva, feathers, environmental samples and urine (additionally, archaeological and museum specimens might be considered minimal-invasive, but see below). These samples are characterized by generally low quantities of host DNA, chemical DNA modifications (degraded and damaged) and often have poor extract qualities (DNA extract contains inhibitors), making standard lab protocols unusable (Monteiro *et al.* 1997; Taberlet *et al.* 1999). Especially, the large proportion of non-host DNA complicates analyses from such samples, as it is inherently difficult to target exclusively true endogenous DNA in a complex mixture of DNA sources.

2.2| Obtaining genetic information from endangered species: PCR amplification-based genotyping

Although DNA sequencing technologies started appearing in the early 1980's (Wu 1972), it took until 1992 for scientists to obtain for the first time genetic information from minimal-invasive samples, exemplifying the challenging task of using such samples (Figure 3). Taberlet & Bouvet 1992 and Höss *et al.* 1992 used snagged hair and faecal samples of brown bear origin in a targeted PCR amplification experiment, and succeeded in amplifying and sequencing mitochondrial DNA. They were also the first to obtain plant dietary information from brown bear faecal samples by amplifying a 365 basepair chloroplast region (Höss *et al.* 1992). Shortly after, a similar method was then used to obtain mitochondrial sequences from a wild chimpanzee population to study social structure (Martin *et al.*

1992) and only a year later, these targeted PCR amplification methods where extended to nuclear DNA, allowing for the determination of the sex of wild individuals from faecal and hair samples (Figure 3) (Taberlet *et al.* 1993).

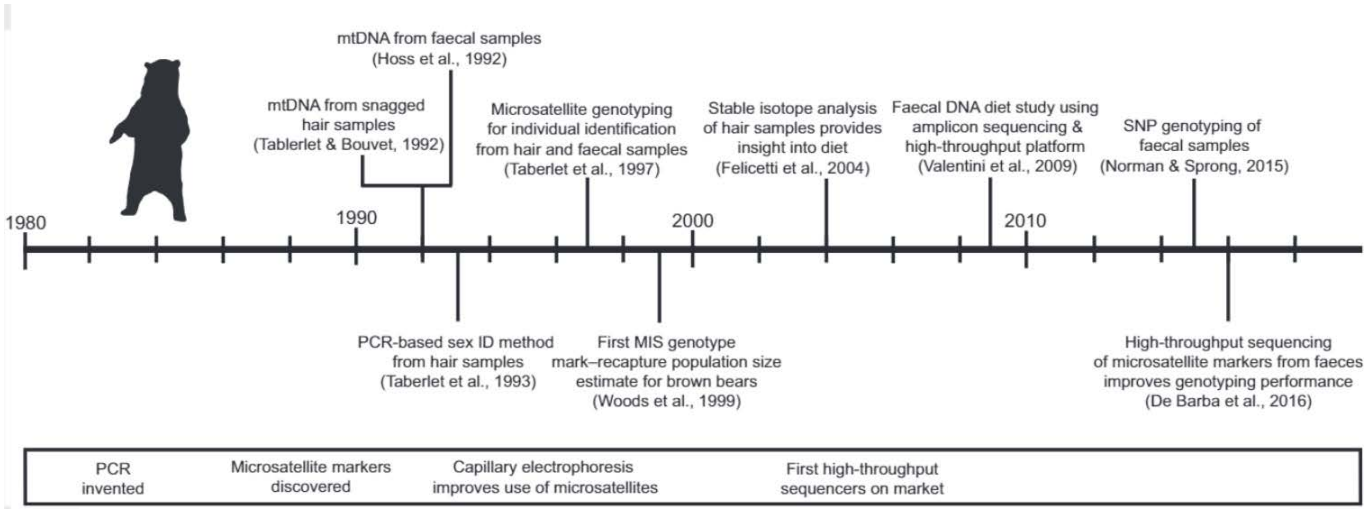

Figure 3. A timeline of developments in minimal-invasive sampling using brown-bear as a model. Figure adjusted from (Carrol et al. 2018)

The possibility to amplify nuclear DNA from minimal-invasive samples and improved understanding of hypervariable nuclear DNA regions such as microsatellites sparked a revolution in conservation genetics in the next years. Accurate identification of individuals and the relationship between individuals through time and space without having to catch, handle or even observe the animals now became a possibility (Figure 3) (Taberlet *et al.* 1997). Individual identifications, based on the amplification of hypervariable (microsatellite[*]) regions, from minimal-invasive samples is such a powerful tool for the study of wild population that it remains widely used until today (Leduc *et al.* 2017; Janecka *et al.* 2017; Baas *et al.* 2018; Ramón-Laca *et al.* 2018; Schultz *et al.* 2018).

The genotyping of individuals from minimal-invasive samples is used for a wide range of applications, such as localising of rare species, estimating demographic history, genetic diversity and gene flow, detecting population structure, migration events and uncover social structure (e.g. Figure 4), detecting

[*] A microsatellite is a 2-6 basepair repetitive DNA sequence, repeated, up to 50 times. Microsatellites occur at thousands of locations within a genome. The very high mutation rate of microsatellites makes them ideal candidates to observe genetic differences between populations and individuals without the need of a large set of markers.

hybridization, monitoring disease episodes, identifying diet items, and recently in wildlife forensics (Schunck *et al.* 1995; Constable *et al.* 2001; Palomares *et al.* 2002; Epps *et al.* 2005; Proctor *et al.* 2005; Kendall *et al.* 2009; Lukoschek *et al.* 2009; De Barba *et al.* 2014; Steyer *et al.* 2016; Balasingham *et al.* 2018; Baas *et al.* 2018).

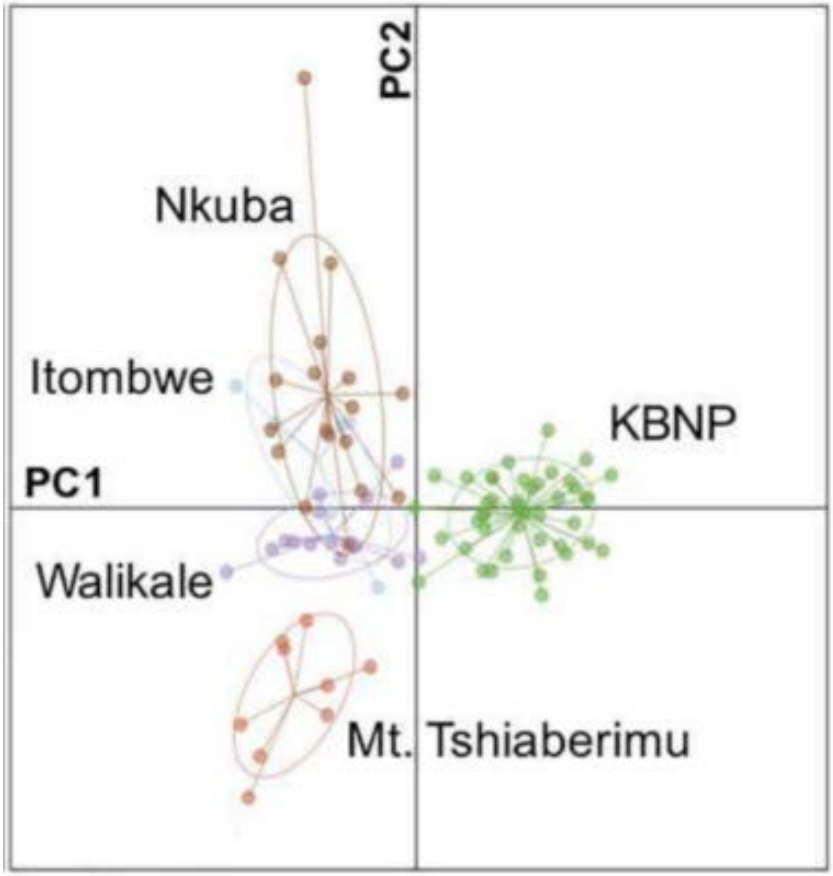

Figure 4. Principal component analysis of eastern lowland gorilla microsatellites profiles. Enough genetic information from minimal-invasive samples (feces) could be obtained to clearly distinguish the different gorilla populations based on their genotypes and obtain estimates of divergence between the (sub)populations. Figure adjusted from (Baas et al. 2018)

Despite these great achievements, already at the onset of minimal-invasive conservation genetics, concerns and limitations of these methods were expressed (Taberlet *et al.* 1999). The DNA quality in minimal-invasively collected samples is often low (degraded), and thus PCR amplifications is restricted to very short fragments (<200-300 base-pairs), limiting the amount of genetic information that can be obtained. Additionally the risk of contamination during sample collection, DNA extraction and DNA amplification is high, given the small quantity of host DNA. Therefore stringent guidelines have to be implemented in order to avoid such contamination (Kwok & Higuchi 1989). Even if great care is taken, (cross)contamination of samples remains a constant danger in PCR-amplification based genotyping

studies (Hoffman & Amos 2004). A further challenge when using PCR-based genotyping is obtaining the correct genotype of an individual. Input quantities are so low (picogram range) that in some cases none or only one of the alleles is present in the PCR-reaction. This leads to "allelic-dropout", which results in biased genetic estimates (Vigilant *et al.* 2001). Thus in order to obtain a reliable genotype multiple replications for each locus are needed (Taberlet *et al.* 1996; Gagneux *et al.* 1997). Some advancements in genotyping methods have helped reducing hands-on time and increased sensitivity (multiplexed PCR amplification), however the strict contamination controls and replication requirements make PCR-based genotyping a labour intensive method and allelic-dropout can never be fully excluded (Arandjelovic *et al.* 2009). Microsatellite-based genotyping also suffers from the "slippage" of DNA-polymerase during amplification, which can give rise to a new allele *in silico* and thus incorrect genotype (Taberlet *et al.* 1996). Finally, the amount of information obtained from microsatellites is limited with respect to detecting fine-scale population structure, identifying ancient gene flow, estimating levels of inbreeding, disease resistance, mutational load and local adaptation. Thus, despite the wealth of knowledge provided by the PCR amplification-based methods from minimal-invasive samples, the field is continuously developing to overcome the above discussed limitations. The final aim in minimal-invasive conservation genetics is to obtain as much sequencing information as what can nowadays be achieved from invasive samples (high coverage whole-genome data). Such methods do not yet exists but multiple efforts are currently ongoing towards this goal. These methods, broadly divided into single nucleotide polymorphisms (SNP) arrays, targeted enrichment and next-generation shotgun sequencing, will be discussed below.

2.3| Obtaining genetic information from endangered species: SNP arrays

SNP arrays that can be used to obtain genotype information from minimal-invasive samples include the Fluidigm assay, Amplifluor SNP genotyping system and MassARRA (Morin & Mccarthy 2007; Gabriel *et al.* 2009). These platforms all provide accurate and automated detection of alleles by amplification of a limited amount of single nucleotide polymorphism (SNPs) (10 to 100) in large sample sizes (usually up to 96 samples per run). These methods have shown to be capable of successfully obtaining detailed genotypes from extremely low input DNA quantities, with similar error rates as microsatellite genotyping, while often having a much higher throughput (Hauser *et al.* 2011; Doyle *et al.* 2016; DeWoody *et al.* 2017). For instance, the Fluidigm and Amplifluor genotyping systems have been successfully used in the genotyping of 523 eagle feather samples at 162 SNPs, 158 wolf urine and scat samples at 192 SNPs and even managed to genotype 16 wild-cats from up to 4 year old dry stored hair samples at 92 SNPs (Nussberger *et al.* 2014; Kraus *et al.* 2015; Doyle *et al.* 2016). Although these platforms hold great promises for higher throughput monitoring of wild-populations, the number of analysis that can be done with this data is, as with microsatellite genotyping, limited due to the relatively small numbers of SNPs.

2.4| Obtaining genetic information from endangered species: target enrichment methods

Arguably the most promising development in obtaining sequence information for minimal-invasive samples comes from improvements in targeted enrichment and capture methods. Such methods aim to maximise the host DNA molecules that become available for sequencing while simultaneously minimising the proportion of "off-target" sequences in the DNA pool. A multitude of target enrichments methods has been developed, but most are similar in that they rely on "baits", short DNA fragments identical (or similar) to the (genomic) regions of interest. These baits are then mixed with the DNA extract obtained from the samples and hybridise with the targeted DNA fragments. These methods frequently make use of magnetic baits, allowing the magnetic capture of the baits while off-target DNA can be washed away after hybridisation. In the next step, hybridised DNA is eluted from

the bait and becomes available for sequencing. Such hybridisation methods hold great promises, as theoretically they allow for an enrichment of complete genomes. Indeed, Perry *et al.* (2010) succeeded in the target enrichment and sequencing of over 1.5 megabases on chromosome 21, chromosome X, and the complete mitochondrial genome from chimpanzee faecal samples. By using capture baits specifically designed to target variable sites in chimpanzee populations, de Manuel *et al.* (2016) succeeded in capturing genome wide data and the complete chromosome 21 from faecal samples, allowing for the identification of population structure and diversity at unprecedented fine-scale from minimal-invasive sampling (Figure 5). Snyder-Mackler *et al.* (2016) extended the existing targeted enrichment method, aiming to obtain whole-genome data. Their method succeeded in enriching baboon faecal samples for up to 40-fold, making some of these samples accessible to low coverage (<1X) whole-genome shotgun sequencing[*]. Recently, a targeted enrichment method initially developed for the use of extracting short host DNA fragments form archaeological specimens (Maricic *et al.* 2010) has been used to obtain whole mitochondrial genomes from eastern gorilla faecal samples, giving a high resolution image of mitochondrial diversity in these critically endangered animals (van der Valk *et al.* 2018).

An extremely promising development in the field of targeted enrichment methods is the use of fluorescence-activated cell sorting (FACS) technology on freshly collected faecal samples. Instead of enriching faecal DNA extracts for endogenous fragments, this method separates “host-like cells” from microbial and plant-like cells before any DNA is extracted. After cell sorting, the DNA can then be extracted from the host-cell enriched extract and subjected to shotgun sequencing (see section 3 for discussion of shotgun sequencing). In some cases the sorted DNA extracts contain high enough endogenous DNA quantities that obtaining high-coverage whole genome data from faecal samples becomes a financially feasible approach (Orkin *et al.* 2018). This method is still in its early days and it remains to be tested how well it performs on different type of samples, collections methods, and how

[*]Shotgun sequencing is the untargeted sequencing of all DNA fragments present in the sequencing library (Quince *et al.* 2017).

the quality of sequence data differs between studies. The first study using this method successfully obtained whole genome data of up to 12X coverage from capuchin monkeys faecal samples (Orkin *et al.* 2018). These are thus the first published whole genomes obtained from minimal-invasive samples with no apparent biases in the distribution of coverage, heterozygosity, or guanine/cytosine content compared to samples from blood or tissue origin (Orkin *et al.* 2018). Obtaining high quality whole genome data from minimal-invasive collected samples thus seems within reach.

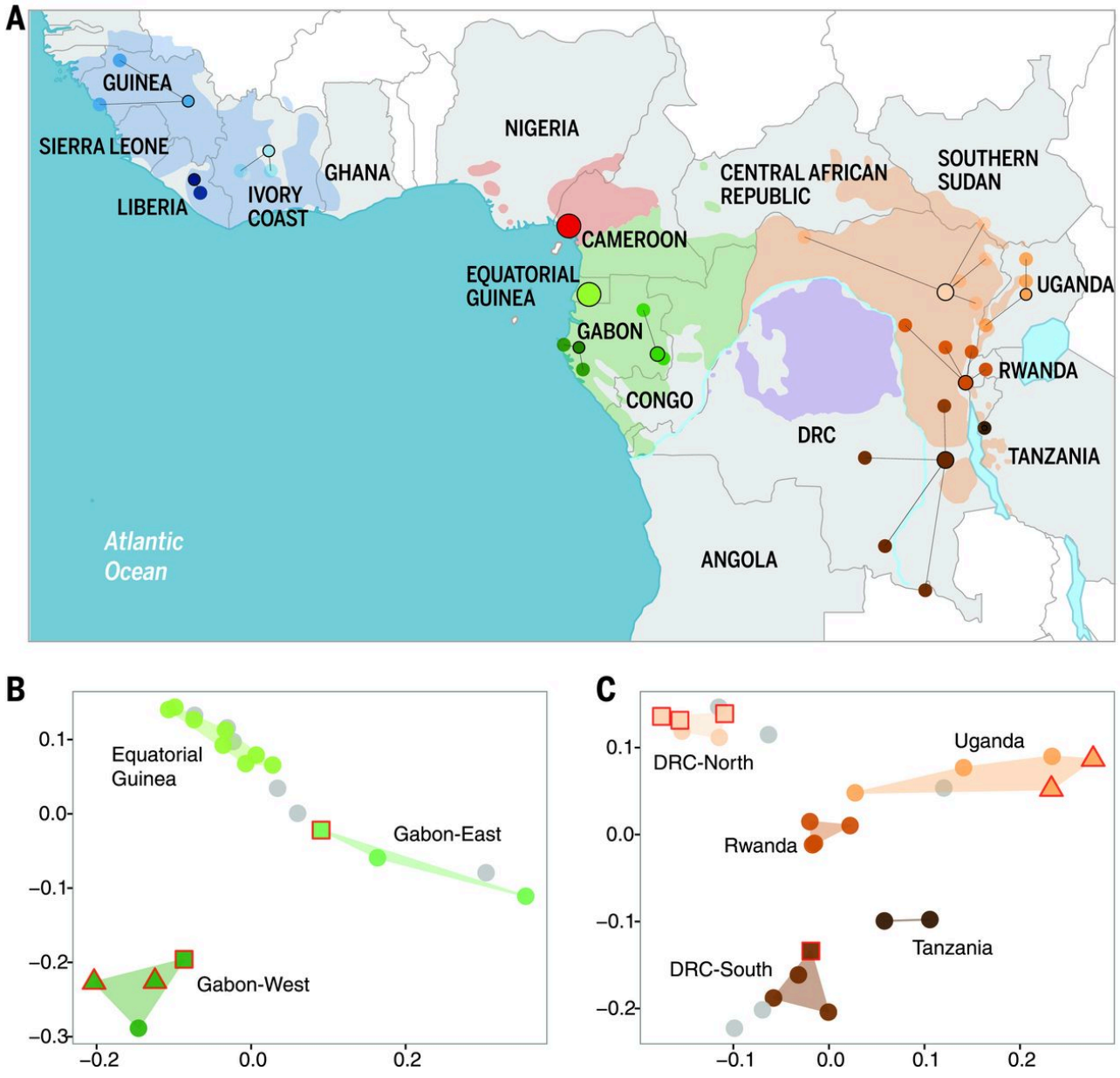

Figure 5. Chimpanzee geography and genetic substructure. A) Geographic distribution of *chimpanzee* populations. Reported coordinates for chimpanzee individuals are shown as circles colored by broad region of origin. (**B** and **C**) PCA plots of chromosome 21 single-nucleotide polymorphism data for (**B**) central and (**C**) eastern chimpanzees. Samples of unknown origin are colored in gray. Circles, high-coverage genomes; squares, low-coverage genomes; triangles, chromosome 21 captured from faecal samples. Figure obtained from (de Manuel et al. 2016).

2.5| Obtaining genetic information from endangered species: shotgun sequencing

Traditionally, conservation biologists have aimed at developing methods that increase the ratio of host to non-host DNA in DNA extracted from minimal-invasive samples (see above). Since most of such samples contain <1% of host DNA, shotgun sequencing such extracts is usually prohibitively expensive, as really deep sequencing is required to obtain sufficient genomic information to make any inferences about the host (Perry *et al.* 2010; Snyder-Mackler *et al.* 2016). However, in some cases, minimal-invasive samples with up to 5% of host-derived DNA have been reported (Snyder-Mackler *et al.* 2016). With ever decreasing cost of sequencing (see section 4) it can be financially feasible to target such samples for shotgun sequencing and obtain complete genomic information. Indeed, whole genome sequences from archaeological samples with as low as 3% endogenous content have already been published (Slon *et al.* 2018). Since, the assessment of endogenous DNA content in a sample requires relatively little resources (e.g. by using qPCR methods), obtaining genomic information by identifying and shotgun sequencing the best samples can be considered in some cases.

Besides obtaining host DNA information, the off-target sequencing data in such studies might contain additional valuable information about the species of interests. For instance, faecal samples can be used to uncover the host diet and microbiome community (see section 3.5). Whereas currently most methods rely on targeted PCR-amplification (16S) or specific capture approaches to study the non-host DNA from minimal-invasive samples, which both suffers from biases in abundance estimations, shotgun sequencing might provide a more unbiased view of the true community composition with the sample (Yildirim *et al.* 2010; Muegge *et al.* 2011)

3| DNA sequencing from a conservation perspective

The field of conservation genomics heavily relies on advancements made in DNA sequencing technology. Such technologies started arising soon after the discovery of the three dimensional structure of DNA in the 1960's (Watson, J. D., & Crick 1953). Since then, DNA-sequencing technologies continues to develop at ever increasing rate, with sequencing output per dollar doubling roughly every 12 months (Figure 9).

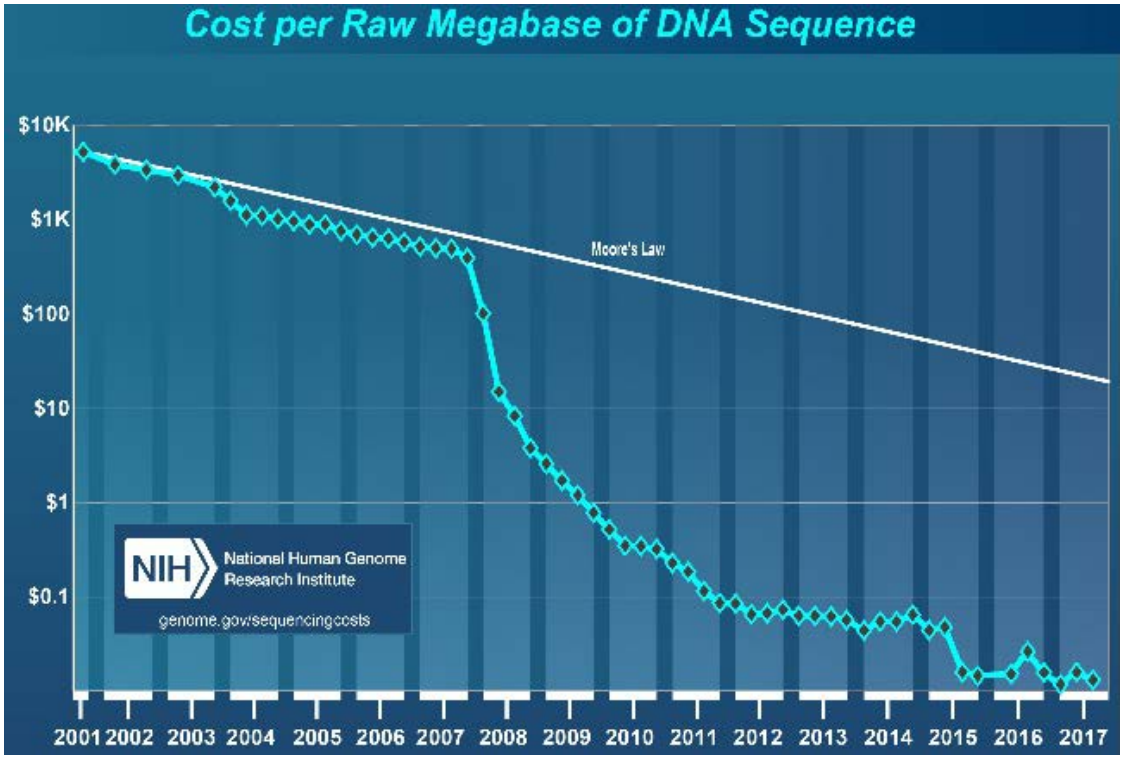

Figure 9. The cost of DNA sequencing. Moore's law describes the increase in computer processor speed per US-dollar (roughly doubling every 12 months). DNA sequencing technologies have developed an order of magnitude faster (note the y-axis log-scale). Figure obtained from the National Human genome research institute.

Current DNA sequencing platforms can broadly be separated into three types: short-read* sequencing (Illumina and BGI-Seq), linked-read sequencing* (10X genomics + Illumina, Bionano maps and Arima HI-C) and long-read sequencing* (Pacific Bioscience and Oxford Nanopore). The introduction of 3rd generation short-read* sequencing platforms in 2007, sparked a true DNA sequencing revolution.

*Short-read sequencing technologies generate sequencing reads of 50 to ~450 basepairs by fragmenting the DNA samples before sequencing. Such DNA fragments are relatively cheap to sequence however have limitations such as reduced resolution for the detection of structural variation and most importantly the necessity of having a reference genome available to map these reads against. Long-read sequencing technologies have generally less data-output for the same costs but can be used for more in-depth analysis and de-novo genome assembly. Linked-reads can either be short (10X genomics)

Within 4 years, the cost of obtaining a "complete" human genome dropped from ~10 million US dollars to less than 10.000 US dollars. Continuous improvements and up-scaling resulted in breaking the 1000 US dollar mark for a complete human genome around the year 2015 (Figure 9). The current cost of short-read DNA sequencing makes large-scale conservation genomic projects now feasible. Multiple conservation oriented studies that included over 40 high-coverage individuals of wolves (Kardos *et al.* 2018), chimpanzees (de Manuel *et al.* 2016) and orangutans (Nater *et al.* 2017) have recently been published. Only a few years ago such studies were financially unobtainable or extremely expensive (Prado-Martinez *et al.* 2013). Multiple large-scale conservation genomic studies are now being performed, such as obtaining high quality genomes for all the 148 remaining Kakapo individuals (Chi 2016). Whereas the cost of Illumina short-read DNA sequencing has not changed in the last few years[*], other sequencing technologies have continued their steep development curve. Most of the recent sequencing developments are aimed at producing long-reads (PacBio, Oxford NanoPore), combined with physical-maps (BioNano and Arima HI-C). These technologies require extremely high molecular weight DNA extracts and high quantities. First, such platforms might have limited applications for conservation genetics from minimal-invasive samples as it is unlikely that such DNA extracts will be able to meet the required quality standards. Second, these platforms are designed for the de-novo assembly of genomes, and although many conservation genomic tools depend on the availability of such a "reference" genome, short-read re-sequencing efforts of populations yield in most cases enough data to answer the conservation relevant questions.

One upcoming long-read sequencing technology that holds great promises for conservation purposes is the Oxford Nanopore sequencing. This is an extremely portable sequencing technology, allowing the real time sequencing of DNA samples in the field (Mikheyev & Tin 2014). The cost per base-pair and error rates of this technology are still too high to be useful for in-depth conservation genomic studies,

or long (Bionano maps and Arima HI-C) and allow for the inference of the physical origin of each read, greatly improving resolution.

[*]This is expected to change in the upcoming years with the introduction of platforms developed by the Bejing genomics institute (MGISEQ-2000 and MGISEQ-200), providing high coverage human genomes for as low as 400 US dollars.

however, this technology has been proven to be useful for real-time species identification and extremely fast disease outbreak monitoring. The portable Oxford-Nanopore sequencing technology was of major value during the 2016 Ebola outbreak, where it allowed for the early and accurate detection of Ebola in the field within 20 minutes (Quick *et al.* 2016). More recently this technology has been used in for instance on-site complete characterisation of glacier microbiota (Edwards *et al.* 2018), real-time biodiversity assessments at broad taxonomic scale in the Hawaii'n rainforest (Krehenwinkel *et al.* 2018), the Ecuadorian Chocó rainforest (Pomerantz *et al.* 2018) and field-based species identification of closely-related plants (Parker *et al.* 2017).

4| Minimal-invasive sampling can provide a wealth of information

Below I will discuss in-depth how genetic data obtained from minimal-invasive samples can help us in a wide range of conservation oriented studies and discuss promising future developments.

4.1| Estimates of relatedness and kin, social, and genetic structure

The understanding of genetic relationships between individuals in a population can reveal novel information about social structure, reproductive strategies, dispersal, and gene flow between populations that is sometimes hard to directly observe or obtain otherwise (Jones *et al.* 2002; Goncalves da Silva *et al.* 2010; Stenglein *et al.* 2011; Caniglia *et al.* 2014; Baas *et al.* 2018). Understanding the social structure of populations can aid in the design of appropriate conservation strategies and is crucial when one aims to minimize inbreeding. In addition, this information may allow the detection of social group genetic structure and inferences on sex-biased dispersal can be made (Bradley *et al.* 2001; Minhós *et al.* 2016). A limited set of markers (as obtained from microsatellites and more recently SNP arrays) already allows for the estimations of the probability that two individuals are related given the data (e.g. parent–offspring). Numerous studies have used minimal-invasive sampling to understand the social and genetic structure of wild populations (see Norman *et al.* 2017 for an overview) and this has, for instance, been crucial in the design of European wild-cat conservation programs (Steyer *et al.* 2016).

4.2| Distribution range, migration, and abundance of endangered populations

The distribution range and population abundances of species are often difficult to estimate. This is especially true for rare or cryptic animals. Using minimal-invasive samples, such as environmental DNA or faeces from unknown individuals can assist in species identification without directly observing the animal. For instance by screening a large number of faecal samples, Stanton *et al.* (2016) identified the presence of okapi in previously un-surveyed regions in the Democratic Republic of Congo (Stanton *et al.* 2016). Such studies are crucial for the optimal allocation of resources towards protected regions and identification of new areas that deserve conservation attention.

Genotypes from minimal-invasive samples have also been extensively used to study individual dispersal. In many cases this is the most efficient way of estimating dispersal distance, as individual identification from direct observations can be unreliable or impractical. Minimal-invasive sampling methods have been applied in for instance monitoring individual movements during reintroduction efforts of brown bears (De Barba *et al.* 2010), wolves (Stenglein *et al.* 2010) and pygmy rabbits (DeMay *et al.* 2017). Genotypes from such samples also provided crucial information on connectivity between different humpback whale habitats (Constantine *et al.* 2014) and roosting related migration in eagles (Rudnick *et al.* 2008). Tracking individual migrations though the use of minimal-invasive genetic data has also been proven of great value in assessing the effectiveness of corridors (Dixon *et al.* 2006) and identifying barriers that potentially restrict gene flow (Epps *et al.* 2005; Kendall *et al.* 2009).

A widely used method to estimate species abundance is by "capture" and "recapture" (as identified by the sample genotypes) of the same individuals through space and time. Such information allows for direct estimates of population size, species density and demographic parameters using statistical methods. This is widely applied to obtain census size estimates and growth rates in endangered animals such as gorillas, Sumatran orang-utans and southern right whales (Taberlet *et al.* 1997; Guschanski *et al.* 2009; Kendall *et al.* 2009; Nater *et al.* 2013; Roy *et al.* 2014; Carroll *et al.* 2018). Whereas historically individual identification was achieved with a small set of markers (mostly PCR-based microsatellite genotyping), more recent methods such as direct microsatellite sequencing or direct SNP analysis now allow more precise-scale individual identification (Fitak *et al.* 2016; De Barba *et al.* 2017; Orkin *et al.* 2018) and open the door for more accurate and detailed methods of population size estimates, such as close-kin mark recapture models (Bravington *et al.* 2016).

4.3| Estimating genetic diversity

A widely used measure in conservation is genetic diversity, which is directly linked to fitness (Reed & Frankham 2003) and thus of major conservation importance (McNeely *et al.* 1990). Minimal-invasive sampling can be used to obtain reliable estimate of genetic diversity in a population based on allelic

diversity and heterozygosity. For instance, large difference in diversity between the different gorilla subspecies were first noticed using such methods (Field *et al.* 1998; Bradley *et al.* 2000; Lukas *et al.* 2004) and only much later confirmed by high-quality whole genome data (Prado-Martinez *et al.* 2013). Microsatellites are well suited for allelic diversity estimates as they are often highly variable and thus a few markers can reveal diversity differences between populations. SNPs have comparatively few alleles (usually only two) and thus many more markers are needed to obtain an estimate of heterozygosity. However, using a high number of SNPs has shown to provide more precise and less biased diversity estimates compared to microsatellites (Doyle *et al.* 2016). Although estimates of genetic diversity from minimal-invasive samples are widely used, current methods are limited in their scope. Genetic diversity is not equally distributed along the genome, and diversity at for instance immune genes might be more important than diversity at putatively neutral sites. For conservation purposes, identifying the most genetically diverse population based on neutral markers and allocate resources based on those inferences might thus not always be the best strategy. Additionally, accurate inbreeding measures such as by identifying stretches of the genome in complete homozygosity or estimates of variation in gene copy numbers are important measures of population variation (Leroy *et al.* 2018) but in most cases require full genome information, not yet feasible to obtain from minimal-invasive samples (however see Orkin *et al.* 2018).

4.4| Identifying hybrid individuals and introgression

Hybridisation and introgression rates have increased dramatically worldwide due to human-induced factors (Allendorf *et al.* 2001). Human actives contributing the most to increased hybridisation rates are the introduction of plants and animals into new ranges and the modifications of habitats (Rhymer & Simberloff 1996). In many cases, hybridization and introgression are major threats to population and species persistence, especially if one of the species is much more abundant than the other (Rhymer & Simberloff 1996; Allendorf *et al.* 2001). It has for instance been suggested that hybridisation between Neanderthals with the much more abundant Homo sapiens drove Neanderthals towards

extinction (Kolodny & Feldman 2017). Genetic monitoring through the use of minimal-invasive sampling can be used for the early detection of hybridisation in the wild. This has been successfully applied in, among others, grey wolves (Monzón *et al.* 2014; Caniglia *et al.* 2014; Kopaliani *et al.* 2014; Godinho *et al.* 2015; Kraus *et al.* 2015), eastern wolves (Benson *et al.* 2012), red wolves (Adams *et al.* 2003; Bohling *et al.* 2016) and European wildcats (Figure 6) (Nussberger *et al.* 2014; Anile *et al.* 2014; Oliveira *et al.* 2015; Steyer *et al.* 2016). The early detection of hybrid zones through minimal-invasive samples can thus assist in taking appropriate conservation measures minimising "extinction by hybridisation" threats.

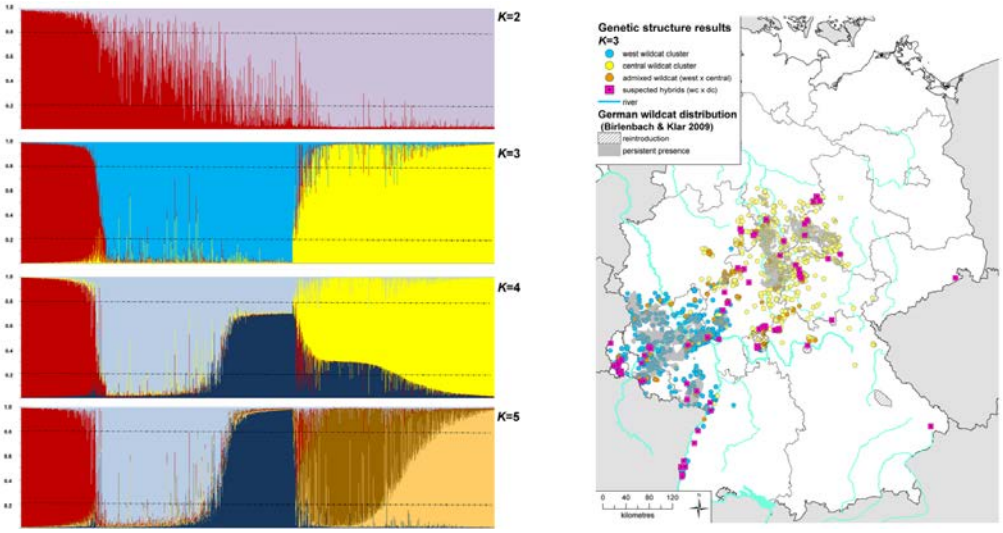

Figure. 6 Detecting hybrid European wild-cat individuals using minimal-invasive samples. Left panel shows genetic substructuring of cat samples (Structure plot) from Germany and Luxembourg using a minimum of eleven microsatellite loci for 2220 cat individuals. **Right** panel displays Structure results for *K* = 3. Admixed individuals between both wildcat clusters are marked orange, whereas samples from potential hybrids of wildcat and domestic cat are displayed as *pink rectangles*. Different colour schemes for the left and right figure are used. Figure adjusted from (Steyer et al. 2016).

4.5| Off target DNA to study diet, gut-microbiome, and parasite infections

In most cases minimal-invasive samples contain large amounts of non-host DNA. Depending on the sample type, such "off-target" DNA is comprised of for instance plant or animal residues from the host diet, or microbes from the gut or skin. Such DNA can be mined to obtain ecological information about the species of interest that can be useful for conservation purposes. The targeted amplification of specific barcoding loci* combined with high-throughput sequencing of faecal samples has proven to

* targeted amplification of samples is achieved by using universal PCR primers to mass-amplify DNA Barcodes from collections of organisms such as present in faecal or environmental DNA extracts.

be an effective genetic monitoring tool to characterise diet (Valentini *et al.* 2009). One of the first studies applying such an approach yielded novel insights into the diet of the leopard cat (Shehzad *et al.* 2012). Since then, metabarcoding approaches have been used to noninvasively study diet in a diverse range of species including Adelie penguins (Jarman *et al.* 2013), golden-crowned sifaka (Quéméré *et al.* 2013), subterranean rodents (Lopes *et al.* 2015), tapir (Hibert *et al.* 2013), brown bears (De Barba *et al.* 2014; Elfström *et al.* 2014), African herbivores (Kartzinel *et al.* 2015), Hawaiin tree snails (O'Rorke *et al.* 2015; Price *et al.* 2017) and red deer (Fløjgaard *et al.* 2017). An extensive study on primate gut-microbiome using faecal samples of captive and wild-born individuals showed a strong shift in the microbe abundances of captive primates ("humanisation of the gut-microbiome") versus wild individuals (Clayton *et al.* 2016), which can have direct conservation consequences in, for instance, re-introduction of individuals (Figure 7). Metabarcoding approaches have technical limitations and therefore diet inference with these methods is semi-quantitative (Deagle *et al.* 2010; Pompanon *et al.* 2012; De Barba *et al.* 2014)[*]. However, the ability to accurately identify primary dietary components is of great importance for ecological studies and conservation.

Additional to dietary characterisations, shotgun sequencing of minimal-invasive samples such as feces can be used to identify potential pathogens (Srivathsan *et al.* 2016). Such methods thus allow for the monitoring of spread of infectious diseases, identifying the most common pathogens within endangered populations and identify infected individuals. Such information will likely be important in the future for determining main population threats (Clare 2014).

[*]Although see (Thomas *et al.* 2014, 2016) for advances in quantitative methods of studying diet in wild-populations

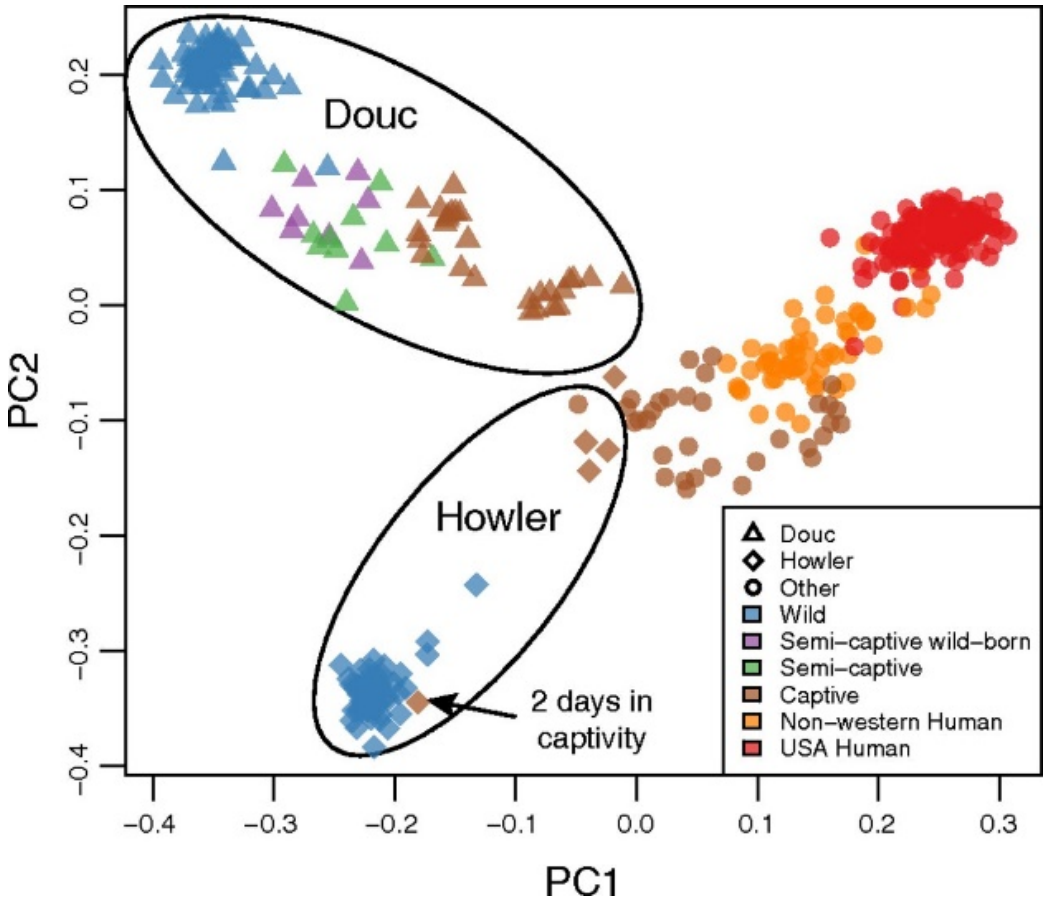

Figure 7. Primate microbiome obtained from faecal samples. The captive microbiome converges toward the modern human microbiome. Although in wild populations the douc and howler microbiomes are highly distinctive, captivity causes them to converge toward the same composition. Semicaptive doucs (green) fall in between wild and captive doucs along the same axis of convergence. The axis of convergence continues toward non-Westernized human populations (Malawi and Venezuela), and finally to the modern US human microbiome. Semicaptive doucs born in the wild have similar microbiomes to their captivity-born counterparts, indicating that transition to captivity from the wild is sufficient to produce the captivity-related microbiome. Figure obtained from (Clayton et al. 2016)

4.6| Off-target DNA to study health related microbial communities

The microbial communities living on or in hosts (microbiome), are a rich area of study in humans and increasingly in wild animals. It has been shown that the health of host individuals is directly affected by these microbial communities through different mechanisms. The microbiome can improve host-resistance to diseases through competitive exclusion or by stimulating immunity responses. Additionally, metabolism and development are directly influenced by microbial communities present with the host (Bahrndorff *et al.* 2016). Such processes can be of high relevance for species conservation. For instance, it has been shown that the skin microbiome of frogs, through the production of fungus protecting metabolites, can aid in the resistance against *Batrachochytrium*

dendrobatidis, a chytrid skin fungus causing massive declines of worldwide amphibian populations that already can be linked to 200 amphibian extinctions (Loudon *et al.* 2014; Jani & Briggs 2014; O'Hanlon *et al.* 2018). The skin-microbiome can additionally contain known pathogens and thus influence an individual's health. Long-term, minimal-invasive monitoring of the southern resident killer whale population in North America showed that antibiotic-resistant bacteria were present in the respiratory microbiome of apparently healthy individuals (Raverty *et al.* 2017). It has also been show that these killer whales occasionally migrate to warmer waters, regenerating their skin and thereby changing the skin-microbiome into a putatively healthier state (Hooper *et al.* 2018). Thus, using minimal-invasive samples to monitor the presence of both beneficial and harmful microbes and alterations in the microbiome over time might reveal changes in an individual's health or be indicative of the environment quality (Amato *et al.* 2013; Tung *et al.* 2015).

4.7| Archaeological and museum specimens to quantify temporal changes

Archaeological and museum specimens harbour genetic information from the past, which allows for the direct quantification and observation of genetic changes through time ("evolution in action"). For extensive reviews on the use of archaeological samples for the study of evolutionary processes see (Wandeler *et al.* 2007; Bi *et al.* 2013; Díez-del-Molino *et al.* 2017; Nogués-Bravo *et al.* 2018). Here I will summarise how such samples can aid in improved conservation practices.

First, it can be debated if the use of museum specimens should be considered as minimal-invasive sampling, as most museum collections reflect the massive killings of flora and fauna by humans. Many animal populations are nowadays extinct or critically endangered due to past trophy hunt expeditions (Batavia *et al.* 2018). Although obtaining genetic information from the already collected archeologically samples will not further impact current-day populations, it is important to be aware of the dark history and ethics associated with such samples (see Arbour & Cook 2006; Elliott 2009; Kaufmann & Rühli 2010 for discussions on the ethics related to archaeological museum specimen).

Since the first advances in the recovering and sequencing of ancient DNA fragments it was recognised that the vast natural history collections in museums worldwide provide an enormous and unique genetic resource (e.g. Bi *et al.* 2013). The extensive demographic declines that endangered species have gone through occurred mostly during the last few decades (Dirzo *et al.* 2014). Consequently, many museum collections contain specimens that pre-date the onset of the most severe anthropogenic-driven declines. Such information is of high value for conservation genomics, as these collections can be used to establish baseline levels of genome-wide diversity. Temporal sampling thus allows for a direct quantification of anthropogenically-driven loss in genetic diversity and increased inbreeding within the last decades, measures that are hard or even impossible to infer from modern genome data (Díez-del-Molino *et al.* 2017). Additionally, museum collections often harbour samples from locations where the species is nowadays extinct. This allows researcher to observe historically present genetic structure or study local adaptations within a species. Museum specimens have for instance been used to show that nowadays extinct eastern gorilla populations harboured unique genetic variants, not contained within the modern population, emphasising the importance of protecting “outskirt” populations in this species (Figure 8) (van der Valk *et al.* 2018).

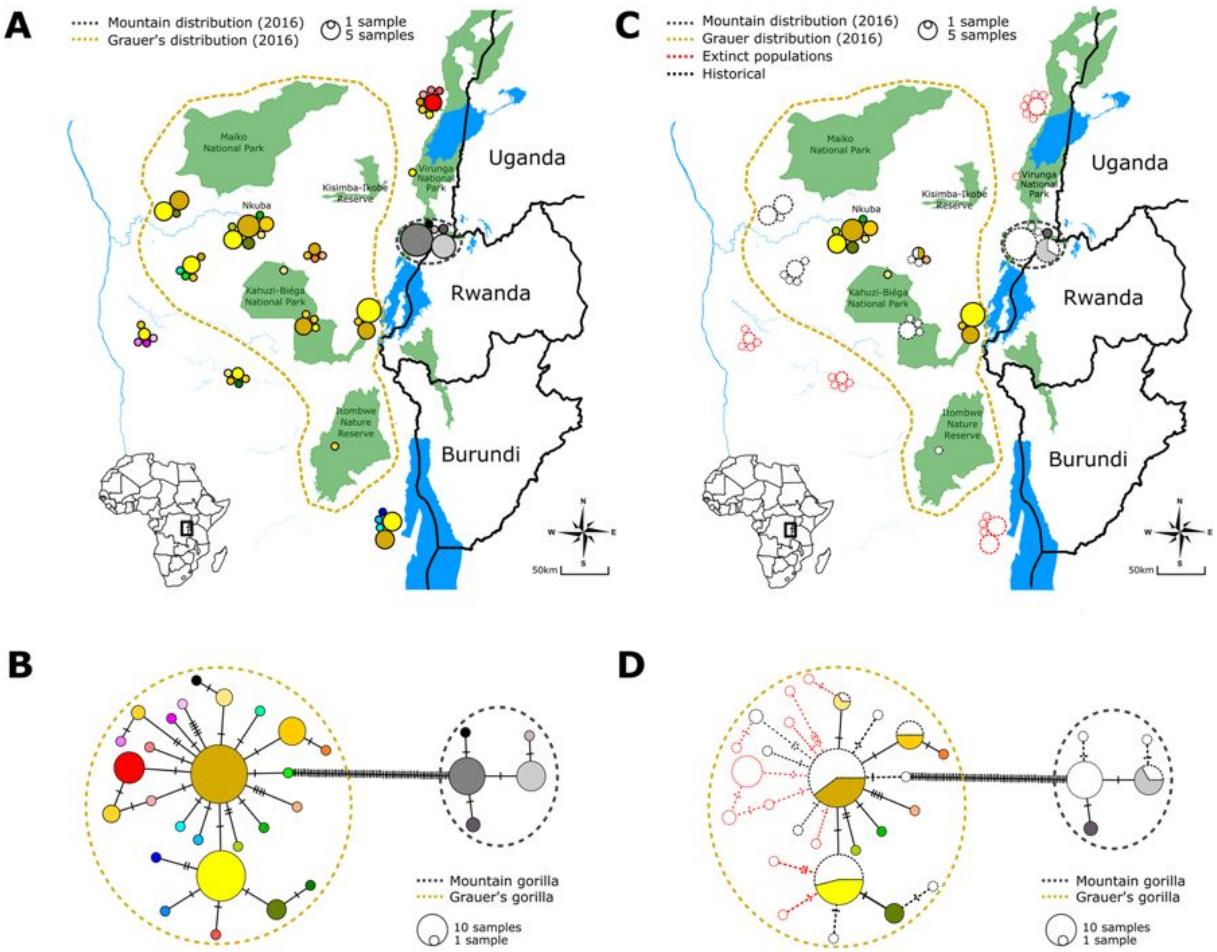

Figure 8. Haplotype map and haplotype network showing the geographic and genetic placement of haplotypes for both historical and modern samples. (**A**) Geographic location of all samples and (**B**) the corresponding mtDNA haplotype network. (**C**) Geographic location and (**D**) the corresponding mtDNA haplotype network of modern samples (colored) and historical samples (shown as outlines). Red outlines designate historical samples from locations outside the current distribution range, where Grauer's gorillas are extinct today. It can be seen that historical haplotypes from extinct populations are absent from the current day population. Figure obtained from (van der Valk et. al 2018)

4.8| Archaeological and museum specimens to study adaptations to changing climate

Besides quantifying genetic diversity over time and detecting past population structure, temporally spaced samples also allow the direct study of adaptations to climate change. This allows us to improve the knowledge of how and when species can adjust to changing climates. Such information can be crucial for the optimal allocation of conservation resources as it can help in predicting species responses to the ongoing climatic changes. Which species can adapt to fast changing climates and how is an ongoing debate. There are now several examples of adaptations that allowed a population of a

species to change its niche and thereby resisting the changing climate. For example, mutations in the woolly mammoth haemoglobin where likely adaptive, allowing this species to exist in high-latitude cold environments during the Pleistocene (Campbell *et al.* 2010). Examples of more recent climate change-induced adaptations are genetic changes that code for a different body colour of owls due to warmer winters (Karell *et al.* 2011), genetic changes coding for white or brown fur in a range of vertebrates adapting to less snowy environments (Mills *et al.* 2018) and the body size shrinkage of Soay sheep of St. Kilda (Ozgul *et al.* 2009).

It is known from the archaeological record that the main response of species during climate change are range shifts (Huntley & Webb 1989). Overall, there is strong evidence of very rapid range shifts and community reshuffling (Tinner & Lotter 2001) in some species, whereas others are lagging behind during fast changing climates (Normand *et al.* 2011). Using archaeological specimens (or ancient sediment DNA) can reveal the presence of species through time, enabling us to understand the timing and speed of such range-shift. This will enable us to understand how such range shifts differ between species, localities and through time.

If species do not adapt to climate change or colonise suitable habitat elsewhere quickly enough, they become extinct. Several mass extinctions events have been linked to change in climate (Barnosky *et al.* 2011; Harnik *et al.* 2012). Using a wide range of ancient DNA samples, the recent extinction of large mammals in the past 50.000 years has been tightly linked to climatic changes (Cooper *et al.* 2015). However, evidence for recent climate-driven species extinctions is limited (Young *et al.* 2016). This might partly be due to the fact that many extinction events remain unnoticed. Ancient DNA can allow us to detect previously unknown species presence and nowadays extinct populations. Such methods have already been used to show that during the last glacial maximum a reduction in overall primary productivity caused losses in genetic diversity and populations of large grazers (Lorenzen *et al.* 2011), depleted killer whale lineages (Foote *et al.* 2011), and contributed massively to local and global extinctions (Cooper *et al.* 2015).

5| “Moon-shot” initiatives

The advances in sequencing technologies and analysis tools have sparked some highly ambitious projects (“moon-shot initiatives”), aimed at revolutionising the field of conservation genomics. Probably the most significant of these projects are the genome10K and 10KP, which aim to produce “near-error-free” genomes of all vertebrates and 10.000 plant species respectively within the next 5 to 10 years (Koepfli *et al.* 2015; Cheng *et al.* 2018). These initiatives have already generated important resources for conservation genomic purposes and are driving the field towards large scale genomic studies. Virtually all conservation genomics project rely on the availability of high quality reference genomes for the design of species specific primers (crucial for microsatellites or SNP array studies), designing target hybridization capture probes or to be used as reference for the mapping of short sequencing reads from population re-sequencing studies. Thanks to these highly ambitious projects, all vertebrate species will soon have a reference genome of either itself or a closely related species[*] available for the use in conservation genomic studies within the next years.

Another set of highly ambitious projects that might prove of great value for future species conservation are the frozen zoo initiatives. Originally proposed by Benford (1993), frozen zoos are storage facilities in which genetic materials taken from animals is stored at very low temperatures. These can either be frozen egg and/or sperm cells or tissue cells reprogrammed into stem cells. A collection of such cells theoretically protects the genetic diversity of the gene pool in declining species. The preserved gene-pool could thus be used to facilitate species recovery during reintroduction or when anthropogenic pressures have decreased and a population starts recovering again. In theory, this would allow the protection of species that are currently entering a bottleneck from the negative genomic consequences associated with such bottlenecks (Benford 1993). Additionally, these cryogenic-preserved cells might be used for the de-extinction of species once suitable habitat becomes available

[*] For most genomic analysis tools, the reference genome of a closely related species (less than ~20 million years divergence time) can be used, if the reference genome of the studied species is not available

again. Such efforts are for instance already ongoing for the Tasmanian tiger and the mammoth (Sherkow & Greely 2013; Jørgensen 2013; Ramaswamy *et al.* 2015; Saragusty *et al.* 2016). It has recently been shown that cryopreserved somatic cells from the functionally extinct northern white rhinoceros contain relatively high diversity, indicating that a de-extinction program of this rhinoceros species might be fruitful (Tunstall *et al.* 2018). Lastly, cryopreserved cells of nowadays critically endangered species might in the future be the only high-quality DNA samples available, and can thus turn out to be of crucial value in future comparative genomic studies. Currently, The San Diego Frozen Zoo stores cell-culture samples of over 800 species and multiple frozen zoos across the globe are in development.

6| Concluding remarks

We are currently in the midst of the Sixth Mass Extinction. The associated anthropogenically-driven worldwide decrease in biodiversity already has pronounced impact on human well-being. It is forecasted that human population growth continues in the next decades, reaching around 10 billion by the year 2050. It thus becomes crucial to increase our conservation efforts, as otherwise anthropogenically-driven extinctions and population decline rates are likely to further increase. Although the importance of biodiversity is nowadays widely recognised by the international community, conservation investments are still below one tenth of the required estimates. Additional to the need for increased investments, new tools that aid in increasing conservation efficiency are urgently needed. The use of genomic methods in combination with minimal-invasive collected samples is likely to be a crucial tool for successful long-term preservation of biodiversity as such methods allow the uncovering of a wealth of conservation relevant information. Genomic methods can assist in the characterization and detection of genetic diversity (one of the three forms of biodiversity), inbreeding depression, species habitat and range, infectious disease outbreaks and dietary changes.

However, the implementation of genomic data into conservation programs is far from straightforward. Much improvements on data quality and quantity obtained from minimal-invasive samples and

analytical methods able to characterise conservation relevant statistics is needed. Exciting new technologies and "moon-shot" projects with these aims, possibly revolutionising the field of conservation genomics, are currently ongoing. Our goal should be to convert these into clear examples of genomic-based conservation success stories, as this will strengthen the public trust in such methods. Ultimately, efficient species conservation will rely on the integration of political, financial, social and scientific approaches, where I foresee a prominent role for genomic data to assist in future conservation efforts.

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