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THE ECOLOGY OF DE-EXTINCTION

Pathways to de-extinction: how close can we get to resurrection of an extinct species?

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Summary

1. De-extinction, the idea that extinct species might soon be resurrected, receives considerable attention in both popular and scientific literature, in particular with regard to its potential ecological and ethical consequences.

2. Here, I review the three main pathways that are being considered at present for de-extinction: back-breeding, cloning via somatic cell nuclear transfer (SCNT) and genetic engineering. I present the state of the art in each pathway and discuss the limitations of each approach as a mechanism to resurrect extinct species.

3. Back-breeding aims to concentrate ancestral traits that persist within a population into a single individual using selective breeding. In back-breeding, ancestral phenotypes may be resurrected after many generations, but the genes that underlie these phenotypes may differ from those that were present in the extinct species.

4. Cloning aims to create genetically identical copies of an extinct species from preserved somatic cells. These somatic cells are fused with egg cells from a closely related and living donor species, which causes cellular reprogramming and embryogenesis, a scientific process known as SCNT. The developing embryo is then brought to term within a surrogate host. Because biological remains degrade post-mortem, cloning of long-dead organisms is not likely to be feasible.

5. Genetic engineering aims to edit the genome sequence within cells of living species so that these genome sequences closely resemble that of a closely related extinct species. This approach draws on recent advances in both ancient DNA and genome editing technologies and is a particularly promising approach to de-extinction. After the genome of a living cell is edited, that living cell can then be used for SCNT.

6. Because the phenotype of an organism is the consequence of the interaction between its genotype and the environment in which it develops and lives, even species with cloned nuclear genomes will not be exact copies of the extinct species on which they are modelled. We should therefore consider de-extinction as a means to create ecological proxies for extinct species.

Key-words: ancient DNA, cloning, de-extinction, genome engineering, paleogenomics, somatic cell nuclear transfer

Introduction

Over the last 5 years, de-extinction, the idea that extinct species may soon be brought back to life, has received increasing attention in both scientific and public arenas. Discussions about de-extinction tend to concentrate on the ethical and political implications of resurrecting extinct species (Sherkow & Greely 2013; Carlin, Wurman & Zakim 2014; Donlan 2014; Friese & Marris 2014) and, increasingly, to focus on the ecological consequences of releasing resurrected species into the wild (Seddon, Moehrenschlager & Ewen 2014). While these are important topics to consider, relatively less attention has been paid to the process of de-extinction itself, specifically whether the technology is sufficiently advanced to bring an extinct animal species back to life.

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Here, I will describe the state of the art for the technologies that are the main foci of existing animal de-extinction efforts. While much of this special feature considers the latter phases of de-extinction, which will include the release and management of any resurrected species, I will concentrate here on phase one, the creation of living animals that can act as proxies for species that are extinct. The three main approaches to achieve this goal are back-breeding, cloning and genetic engineering (Shapiro 2015). While each of these approaches has shown promise with respect to de-extinction, significant challenges remain. Ultimately, the feasibility of phase one will vary depending on the species itself, with some species being technically simpler to resurrect than others, and on the end-goal of the de-extinction project. While it is likely to become feasible to resurrect extinct phenotypes, it may never be possible to create an identical living copy of an extinct species. These technologies should therefore be considered as means to create *ecological proxies* for species that are no longer alive, which may benefit ecosystems, for example by restoring critical interactions among species (Shapiro 2015; Corlett 2016; IUCN/SSC In press) (Fig. 1).

Back-breeding

Back-breeding is the term used to describe the use of selective breeding to resurrect specific ancestral traits within populations of living organisms. Similar to traditional selective breeding programmes, mating pairs are selected for back-breeding based on their phenotype, for example, if they display a particular morphological or behavioural trait. However, whereas most selective breeding programmes aim to create or enhance new traits, such as selection during domestication for larger fruit sets in plants or tameness in animals, back-breeding aims to resurrect traits that have been lost or diluted over evolutionary time.

Although selective breeding is a powerful way to increase the prevalence of specific traits within a population, back-breeding has several important limitations as an approach to de-extinction. For example, back-breeding requires that the target ancestral traits persist within a living species. This means that the approach may be appropriate only when the extinct species is very closely related to a species that is still living. In addition, there is no certainty that the selected phenotype results from the same underlying genotype – or, more likely, suite of genetic and environmental interactions (Lehner 2013) – that produced the phenotype in the extinct species. Back-breeding may also result in a higher degree of inbreeding within the population or in the creation of disadvantageous combinations of alleles, both of which may reduce the population's overall fitness (Marsden *et al*. 2016).

One of the most high-profile back-breeding projects is to resurrect the aurochs, which is the extinct species that gave

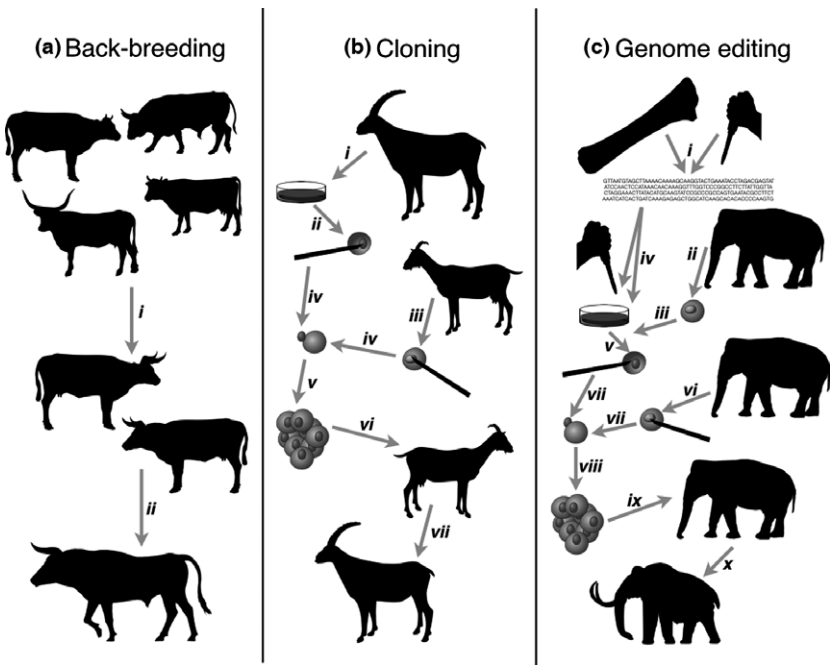

Fig. 1. The three approaches to de-extinction. (a) In back-breeding (*i*), individuals are selected for breeding based on phenotype. (*ii*) After many generations of selective breeding, the extinct phenotype is resurrected. (b) In cloning (*i*), somatic cells are harvested from a living organism and cultured *in vitro*. (*ii*) Nuclei are removed from these cultured cells. (*iii*) At the same time, egg cells are harvested from a closely related species and enucleated. (*iv*) The nucleus from the somatic cell is fused to the enucleated egg, and (*v*) the cell begins to divide. (*vi*) The embryo is implanted into a surrogate maternal host, which (*vii*) gives birth to a genetic copy of the somatic cell harvested in (*i*). (c) In genome editing (*i*), DNA is extracted from the remains of an extinct species and used to sequence and assemble a genome, which is used to identify sequence differences between a closely related living species. (*ii*) Cells are harvested from that close living relative and (*iii*) cultured *in vitro*. (*iv*) Genome editing is used to change the genome sequence of that living cell so that it more closely resembles that of the extinct species. (*v–xi*) are the same as cloning (*ii–vii*).

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rise to present-day domestic cattle (Loftus *et al.* 1994; Edwards *et al.* 2007). Aurochs were extinct by 2000 years ago across much of Asia, Europe and North Africa, but may have survived in isolated populations in central Europe until the 17th century (van Vuure 2005). Based on present-day reconstructions from archaeological remains, cave drawings and historical documents, aurochs were larger than today's cattle, with forward-facing horns and an aggressive temperament (Pyle 1994; van Vuure 2005). These and other traits persist to the present, but are dispersed across many breeds of living cattle. Selective back-breeding can therefore be used to concentrate these traits into a single, new breed of cattle that could share many characteristics with extinct aurochs.

Aurochs were probably the first species targeted for de-extinction and, thanks to recent advances in ancient DNA technologies, are among the best genetically characterized extinct species to date. The first attempt to resurrect aurochs began in the 1920s, when German zoo directors Lutz and Heinz Heck began a back-breeding project that eventually created a present-day breed known as Heck cattle (van Vuure 2005). Heck cattle are aggressive and have a primitive appearance, but are generally not considered as successfully back-bred aurochs as they lack some of aurochs distinctive morphological features (Stokstad 2015). Today, at least three other efforts to back-breed aurochs are underway (Stokstad 2015). These present-day efforts have the advantage of using genetics to guide breeding decisions, with high-quality genome sequences now available from a wide variety of cattle breeds and, recently, from the 7000-year-old fossil remains of an extinct aurochs (Park *et al.* 2015). This ancient genome was used to identify more than 300 genes that may have been selected during the process of cattle domestication. For the purposes of de-extinction, these are ideal target genes to revert to the ancestral, undomesticated state.

Cloning

Cloning refers to the technique known as somatic cell nuclear transfer (SCNT; Wilmut *et al.* 2002) to create an exact genetic copy of a living organism. In SCNT, the nucleus from an adult somatic cell is injected into an enucleated egg cell and then reprogrammed by the host egg cell. This reprogramming reverts the somatic cell into an undifferentiated pluripotent stem cell, which can then develop in the same way that an embryo would develop following fertilization of an egg cell by a sperm cell. The organism born after SCNT will have an identical nuclear genome sequence to the donor of the somatic cell.

Since the successful cloning of Dolly the sheep in the mid-1990s (Wilmut *et al.* 1997), technical improvements and better understanding of cellular reprogramming have made the approach increasingly efficient (Ogura, Inoue & Wakayama 2013; Verma *et al.* 2015). Still, while some studies report high success rates, on average, <5% of potential clones develop into live offspring (Whitworth & Prather 2010). Intriguingly for the purposes of de-extinction, interspecies cloning, where clones of one species are born to maternal surrogates of a different species, is also possible (e.g. Lanza *et al.* 2000). However, evolutionarily close relatives may be necessary for this to be successful (Loi, Galli & Ptak 2007).

Cloning is an attractive approach to de-extinction because, unlike back-breeding, the resulting organism will be identical, at least at level of the nuclear genome, to the extinct donor of the somatic cell. Cloning, however, requires intact living cells, which are not available for most extinct species. When an organism dies, its tissues and the DNA within those tissues begin to decay almost immediately. Intracellular and environmentally derived nucleases gain access to and degrade the DNA, and physical and chemical processes, including oxidation, hydrolysis and radiation, cause further damage by inducing DNA cross-links, nucleotide modifications and DNA-strand breaks (Lindahl 1993; Dabney, Meyer & Paabo 2013). Because the repair mechanisms that have evolved to correct such damage during life no longer function after death, the damage accumulates, resulting in increasingly fragmented and damaged DNA.

Fortunately for very recently extinct species, it is possible to create clones from cells that have been collected, cultured and frozen prior to death. In 2003, a cloned calf of a bucardo, an extinct subspecies of Spanish ibex, was born (Folch *et al.* 2009). Several years earlier, a team of Spanish, French and Belgian scientists captured and harvested a skin sample from the last living individual of this subspecies, a female called Celia. Fibroblasts from this skin sample were grown in culture and then frozen in liquid nitrogen (Folch *et al.* 2009). These frozen cells were later thawed and then used for SCNT with enucleated (nucleus removed) eggs from domestic goats. While most of the reconstructed embryos did not develop, a single female bucardo was born. Unfortunately, this animal had a lung deformity that resulted in its death just after birth. Nonetheless, this work represents the only de-extinction by means of SCNT to date; unfortunately, the organism born from this process was not viable.

While the bucardo work benefits from the availability of intact and living (frozen) cells, it may in some circumstances be possible to create clones from cells that are less well preserved. In Loi *et al.* (2001) cloned a mouflon, an endangered subspecies of wild sheep, from a non-viable cell harvested from an animal that had been found dead in a field. The cell used in this experiment was non-viable (it could not be revived and coaxed to divide), and yet its fusion with an enucleated egg resulted in embryogenesis. This result hinted that freezing and desiccation, which are processes that promote the long-term preservation of biomolecules within organismal remains (Lindahl 1993) but also lead to lysis of the nuclear membrane and therefore cellular non-viability, may nonetheless provide cells that can be used for SCNT. By 2008, several independent efforts had proven this hypothesis: cloned mouse embryos

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were created from freeze-dried cells (Loi *et al.* 2008; Ono *et al.* 2008), from cells that were frozen for nearly a year (Li & Mombaerts 2008) and from cells taken from mice whose entire bodies had been frozen for up to 16 years (Wakayama *et al.* 2008). To get around the problem of nuclear membrane lysis, these teams injected entire cells directly into enucleated eggs. Those egg cells that began to divide were then made into lines of embryonic stem cells, and these cells were then used for SCNT.

The results to date suggest that clones of some recently extinct species might be obtainable; however, several challenges remain. First, in the studies that used frozen (archived) cells, the efficiency of embryo generation declined with the amount of time that the cells had been frozen (Wakayama *et al.* 2008). This bodes poorly for species that have been extinct for many hundreds or thousands of years, even if their remains are rapidly desiccated or preserved in frozen sediment. Secondly, the conditions of freezing for the mouse experiments were held constant through time. Even animals that become buried in frozen soils will experience seasonal changes in temperature, in particular during the period immediately post-mortem. Larger animals will freeze more slowly than smaller animals, leaving more time for enzymatic decay. Mummies, which often have the appearance of being better preserved than isolated skeletal remains, may take longer to freeze through than isolated remains, and microbes released from the gut during this period may cause significant damage (Hess *et al.* 1998). During the summer of 2015, reports surfaced in the South Korean popular press of progress using the whole-cell fusion approach described above to generate mammoth embryos (He-suk 2015). However, no data or official reports have emerged from either laboratory that is supposedly involved with this work, and 16 years (Wakayama *et al.* 2008) remains the oldest frozen specimen of which healthy clones have been generated.

Genetic engineering

The third approach to resurrecting extinct species takes advantage of recent advances in two fields, ancient DNA and genome editing (Doudna & Charpentier 2014; Shapiro & Hofreiter 2014), which together pave what I believe is the most likely route to de-extinction (Shapiro 2015). Advances in ancient DNA extraction and DNA sequencing technologies are making it increasingly feasible to reconstruct full genome sequences from extinct species. These genomes can be aligned to genome sequences from the living species to which the extinct species is most closely related. Once the key sequence differences between the extinct and extant species' genomes are known, genome engineering can be used edit the genome of the living species in cells *in vitro,* resulting in living cells with genomes that express extinct genes. These living cells can then be used for SCNT.

The first step in this pathway is to reconstruct the genome sequence of the extinct species. While cells do not remain viable post-mortem, DNA survives and, despite becoming increasingly fragmented over time, is recoverable from a variety of tissue types. The rate of DNA decay is slower in cold environments than in hot environments. Consequently, early ancient DNA research concentrated on animals that lived and died in arctic regions (Shapiro & Cooper 2003). Cold preservation remains an important criterion for long-term DNA preservation; the oldest specimen from which a genome has been sequenced is a 700 000-year-old horse bone found in frozen soil in the Canadian Arctic (Orlando *et al.* 2013). However, improvements in ancient DNA methodologies within the last several years (Dabney *et al.* 2013; Gansauge & Meyer 2013) have made it possible to reconstruct full genome sequences from samples preserved in an increasingly wide range of preservation environments, and full genomes are now available for several candidate species for de-extinction including mammoths (Lynch *et al.* 2015; Palkopoulou *et al.* 2015), aurochs (Park *et al.* 2015) and passenger pigeons (Hung *et al.* 2014; Shapiro *et al.* 2016).

While having a sequenced and assembled genome is a key to this pathway to de-extinction, not all ancient genomes are equally likely to become available, even as technologies improve. DNA is still challenging to recover from most remains preserved in hot and wet environments, and the oldest remains to contain any preserved DNA are not likely to predate the Pleistocene (Shapiro & Hofreiter 2014). In addition, due to the fragmented nature of recovered ancient DNA sequences, taxonomic biases are likely to affect which extinct species will eventually have their genomes sequenced. Because recovered DNA sequences are very short, technologies that either sequence long DNA molecules [e.g. Pacific Biosciences long-read sequencing technology (English *et al.* 2012)] or that require long molecules to prepare DNA sequencing libraries for short-read sequencing platforms (e.g. 'Chicago' libraries; Putnam *et al.* 2016) are not available to ancient DNA. This means that ancient genomes cannot be assembled *de novo* and instead will be assembled by mapping the recovered short DNA fragments to genomes that have been assembled previously, creating what is called a reference-guided assembly. The greater the evolutionary distance is between the previously sequenced reference genome and the extinct genome, the fewer the molecules will map reliably to that reference (Prüfer *et al.* 2010). Therefore, genomes from extinct species with no close living relatives, such as the New Zealand moa, which diverged from its closest living relative, the tinamou, around 60 million years ago (Phillips *et al.* 2010), may be difficult to assemble correctly and completely.

Once the genome of an extinct species has been assembled, the next step is to identify the parts of that genome sequence that are responsible for the target phenotype. A logical goal might be to change every site in the extant genome where the sequence differs from the extinct genome. This number of changes is likely to be large; for example, the number of fixed nucleotide differences between mammoths and Asian elephants, which diverged

only ~5 million years ago, is around 1·4 million (Lynch *et al.* 2015). Genome editing approaches are improving both in the efficiency with which they target and replace the correct region of the genome (Chu *et al.* 2015; Maruyama *et al.* 2015) and in their capacity to make multiple changes simultaneously (Yang *et al.* 2015). However, the largest number of changes made at once – to date, 62 – is small relative to the total number of differences between species. In addition, while Yang *et al.* used genome editing to knock out (turn off) a retroviral sequence that was present in 62 different places in genome, no team has succeeded in making this many *edits* to a genome sequence in a single experiment, nor is it possible as yet to predict the consequences of large-scale editing on genome stability. For now, therefore, it will be crucial to identify one or a few target phenotypes and design experiments to make the edits that underlie those phenotypes. This, too, presents a challenge to de-extinction. Laboratory experiments with model organisms and comparative analyses of the growing diversity of sequenced genomes are revealing links between genotype and phenotype. However, this work is also showing that phenotypes are typically influenced by more than one and sometimes hundreds of genes (Lehner 2013).

Despite these challenges, progress has been made both to discover extinct genotypes that have known phenotypic consequences and to engineer those extinct genotypes into the genomes of living organisms. For example, Campbell *et al.* (2010) estimated phylogenies for several genes associated with the haemoglobin protein and used these to identify specific mutations at which mammoths and Asian elephants differed. They then inserted the Asian elephant version of these genes into a haemoglobin expression vector and used site-directed mutagenesis to change the Asian elephant sequence into the mammoth version of the sequence, providing a means to test the phenotypic consequences of the mammoth-specific mutations. When the protein complexes were expressed in *Escherichia coli,* the complex containing the mammoth version of the genes was more efficient at carrying oxygen at low temperatures than that which contained the elephant version of the genes, suggesting that the mammoth version may have been useful as mammoths adapted to life in cold climates (Campbell *et al.* 2010). These genes are among fourteen potential cold tolerance genes that a team in George Church's laboratory at Harvard University have successfully engineered into living elephant cells (Callaway 2015). Although the experiments at the Church laboratory are still in progress, this success highlights the power of genome editing approaches to engineer extinct genes and phenotypes into living cells.

When genome editing is complete, the next step in this pathway to de-extinction is to transform the cell containing the edited genome into a living organism. For mammals, this is possible via SCNT, assuming that an appropriate surrogate maternal host can be identified. If successful, this clone can be used to create multiple individuals. However, the genomes of each of these individuals will be identical, which creates a type of founder effect, the consequences of which will depend on the diversity of the population from which the host (edited) cell was taken and the length of time that the newly established population remains small (T. Steeves, unpublished data).

For some species, SCNT is not an option. For example, nuclear transfer is not yet possible for egg-laying species including birds, monotremes or reptiles, for example, because the reproductive physiology of these animals limits access to and manipulation of the very early egg (Kjelland, Romo & Kraeme 2014; Doran *et al.* 2016). One possible solution is to edit germ cells, rather than somatic cells. In birds, primordial germ cells (PGCs), which are the precursors of gametes, can be isolated from developing embryos and cultured and genetically modified *in vitro* (van de Lavoir *et al.* 2006). PGCs with edited genome sequences can then be re-injected into eggs at the appropriate developmental stage, after which they will migrate to the gonads and can be used to create transgenic birds (Macdonald *et al.* 2012; Park *et al.* 2014). This approach, while in its infancy compared to SCNT, has tremendous potential for genetically modifying organisms for which cloning is not an option.

Proxies, not copies

None of the approaches described above will culminate in the birth of an organism that is an identical copy to one that is extinct. In back-breeding, a target phenotype that is achieved may be recovered through a different genetic or gene-by-environment mechanism than that which resulted in the extinct phenotype. Genome editing guarantees that the same gene is responsible for the phenotype. However, that gene will be expressed as part of a different genomic background, the consequences of which cannot be known until the organism is born. In both instances, continued artificial selection might be required to maintain that phenotype in the present-day ecosystem.

Even organisms cloned from frozen cells will not be identical to the extinct organism with which they share their nuclear genome. For example, in SCNT, the mitochondria that are present in the enucleated egg cell are passed on to the developing offspring. Mitochondria have their own genome that encodes for genes involved with cellular metabolism, which is fundamental to life. The products of these mitochondrial genes interact with the products of genes encoded by the nuclear genome. This interaction may therefore affect the phenotype of the clone (Burton, Ellison & Harrison 2006). Perhaps, more importantly, however, are gene–environment interactions that will necessarily differ from those experienced by the extinct species. The clones will develop within the eggs and uteri of a different species, whose diet, environment and even genes (Bird 2007; Li, Zheng & Dean 2010; Teh *et al.* 2014; Dosch 2015) influence the developmental process and resulting phenotype. When the animal is born, it will be raised by a surrogate species, with different behaviours and

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social structures, which will affect its phenotype. It will live in an environment that is different from that which persisted in the past, and consume a different diet than was consumed by other members of its species. It will have a different microbiome, different stressors and, ultimately, a different epigenome than the donor of the somatic cell to which it is genetically identical.

Precise replication of an extinct species, however, is not necessary to achieve the conservation-oriented goals of de-extinction. In the majority of ongoing de-extinction projects, the goal is to create functional equivalents of species that once existed: ecological proxies that are capable of filling the extinct species' ecological niche. Proponents of back-breeding aurochs hope to release these animals into abandoned farmland within what was once the aurochs' range (Stokstad 2015). Proponents of resurrected mammoths hope for something similar: genetically modified elephants that can survive the cold winters in Siberia and functionally replace mammoths on that landscape (Zimov 2005). While the new aurochs and new mammoths will not be genetically identical to extinct aurochs or extinct mammoths, there is no reason to expect that they would not graze, recycle and disperse nutrients, and as such help to maintain a diverse and healthy ecosystem, just as aurochs and mammoths once did.

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Data accessibility

This manuscript does not use data.

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