How science supports fiction, or to which extent technologies have advanced the progress towards the vision of de – extinction

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De – extinction, or the attempt of trying to bring back to the world different animal species which are already gone extinct, is lately gaining more and more acclaim, among others due to the fact, that emerging technologies of genetic engineering are opening a wide range of new possibilities in the field. Starting from the techniques which make the isolation of ancient DNA more effective, through the methods of its amplification and sequencing, to finally discovering the whole genomes, the plan of making de – extinction real is becoming gradually more possible. Successful sequencing of the mitochondrial genome of extinct quagga was a breakthrough, then the time for other species came. Great hopes are placed on restriction enzymes, especially those which work in CRISPR/Cas9 system, which enable us to edit the genomes of living relatives still more efficiently. Apart from genome editing, the methods of selective back - breeding or cell nuclear transfer are used.
Each species requires an individual consideration in the terms of the DNA material available, choosing the optimal method and the likelihood of successful de – extinction. The idea, however, leaves still many questions unanswered on the aspects such as ethics, ecology, funds and society, therefore it is doubtful, if we are ever going to face the full restoration of population once gone extinct.

Introduction

The attempts on bringing extinct species back to life date back to as late as the thirties of the twentieth century, when two German zoologists, brothers Heinz and Lutz Heck, tried, by the method of selective back – breeding, to restore the population of European aurochs (*Bos primigenius*) and the tarpan horse (*Equus ferus ferus*) [1, 2]. However, the knowledge on genetics at that time was insufficient to call that effort a fully successful one. What is more, to make back – breeding work, one needs appropriate timespan, whereas the two decided to conclude the experiment after a little more than a decade [1, 3a, 4, Tab.1].
De – extinction is a term originally coiled in 2012, which includes widely understood attempts to restore the extinct species in its most ancestor – resembling shape and to restore the whole populations [2]. However, before the term gained its exact definition, the first using of the word was most probably in the book 'The Source of Magic' by Piers Anthony, published in 1979 [3b]. Of similar meaning are, also in use, the extinction reversal or revival. In March 2013, as a result of the co-operation among three non-profit organizations: TED (Technology, Entertainment and Design), National Geographic Society and Revive & Restore, a daylong conference took place, during which the most outstanding scientists held a series of lectures, thus making the de – extinction more popular [2, 3c, 5].

Possible ways

Depending on the species and the time which has passed since it vanished from the surface of the Earth, there is a speculation about the extent to which the following methods are usable: selective back – breeding, cell nuclear transfer and the latter combined with genome editing [6]. The first of the methods, in which science is largely 'relieved' by the evolution, has been used among others in the extinct quagga (*Equus quagga quagga*) case. The project took on in 1987 and currently the fifth generation of the offspring was born, the stripes reduction of which bears undoubtedly a resemblance with the quaggas [7, Fig.1]. What makes the method of back – breeding possible in this case is the fact, that guagga is indeed a subspecies of the plains zebra species [8]. Another example is the plan of restoring aurochs population, driven by the Dutch scientist Henri Kerdijk-Otten [3d]. Cell nuclear transfer makes sense if either the living cells of the species or the living individuals of the species are available [3e]. In the future, the so called frozen zoos could serve as sources of genomes, where in liquid nitrogen the gametes and embryos of endangered species are being preserved nowadays [9]. To many species, however, this approach is not applicable, which means the genome needs to be formerly reconstructed. If so, the starting point could be the so called ancient or fossil DNA (aDNA, fDNA, respectively), which requires special cautions when dealt with [10].

Collecting the material

The first case of working with aDNA was sequencing the mtDNA of the quagga mentioned [8]. According to the fact that the environmental conditions in which aDNA is being preserved are rather poor and there are no repair mechanisms in dead tissues, any attempts of working with aDNA are difficult, and the endogenous DNA found in fossils is mainly less than 1% of the whole DNA extracted from the specimen [11, 12]. Thus, the special protocols of making the process of aDNA isolation from powdered fossils more effective have been introduced. As an example serves the method in which the DNA is bound to silica in the presence of guanidine isothiocyanate, the advantage of which is keeping apart both the DNA and the potential inhibitors of PCR reaction [13]. Another example is the protocol, which presents the use of 'pre – digestion' before the proper enzymatic digestion of the material in the solution which includes among others EDTA. The protocol (in the temperature of 50 Celsius degrees and 1 hour time) helped to increase the ratio of endogenous DNA in the specimens even 2, 7- times when compared with other protocols [14]. The kind of tissue used also plays a role [15, Fig.2]. It has been proven, for instance, that, in comparison with the inner dentin, even 14 times more DNA can be extracted from the outer parts of the tooth's roots [14].

The molecular level

The next step, after having the material isolated, is to amplify it. Before PCR was discovered in 1983, bacterial colonies were used to amplify DNA [3f]. The PCR method, due to its numerous advantages, is widely used, in the field of archaeology, too [16, 17, 3g]. In the case of extinct species, one needs to use the starters of sequence which is equal for all vertebrae or specific for the closest living relative [18]. Also in use are real-time PCR, nested PCR or whole genome amplification [10]. In 2006, with the use of emulsion PCR and SBS method (sequencing by synthesis), it has become possible to exactly indicate both the places in DNA and the types of changes, which the bases in aDNA underwent [19]. A year later the paper was published, in which with the use of CSR method (compartmentalized self-replication),

the polymerases combining the features of three species of genus Thermus have been isolated. Their efficiency, thermal stability and sensibility when applied to aDNA from Pleistocene significantly outran same features in Taq polymerase [20, 21]. In 2007, the method of extending the single primers was introduced (SPEX – single primer extension –based), the vast advantage of which was the ability to differentiate between the mispairing caused by the passing time and the one, which was polymerase's failure [22]. Therefore, the results of the following sequencing were even more reliable.

Troublesome genome

Due to the decay which aDNA undergoes, yet in 2001, to ensure the reliable sequencing, few PCR reactions had to be carried through independently, which was both time – and money – consuming [23]. Hypothetical consideration of cave bear mtDNA, at that time it would have taken tens thousands years of work [24]. Technologies available nowadays make this process reachable in a shorter timespan: in 2008 the next generation sequencing was used to sequence the 80% of wooly mammoth nuclear genome of its hair specimen, and it was at the same time the first nuclear genome ever to have been sequenced from aDNA [25]. However, even the genomes of any living creatures are not sequenced in 100%, due to the fact that there are certain places in the genome not accessible with current abilities, such as heterochromatin or centromeric DNA [3h, 26]. Though the synthetic centromeres of human chromosomes have already been created, putting the genome of a dead individual together is associated with too many difficulties to consider it as a goal real to reach any soon [27, 26, 3i].

Cell nuclear transfer

Somatic cell nuclear transfer (SCNT) is a method commonly known under a less precise name as 'cloning' and which enabled Dolly the sheep to have been born in 1996. Dolly was the first mammal ever to have been born from denucleated somatic cell of an adult living individual [28]. If the intact genomes of extinct species are available, this approach can be as well applied. An example is when in 2003 a mammal called bucardo, a subspecies of Iberian ibex species (*Capra pyrenaica*), was born. The nuclei were taken from the tissues belonging to the last living bucardo female named Celia after she died in 2000. As a surrogate mothers, Spanish ibexes of *Capra pyrenaica hispanica* subspecies or their hybrids with domestic goats were used. In merely one case the pregnancy came to term, but the baby bucardo unfortunately died minutes after birth due to lung malformation [29]. Similar projects had been undertaken before the described situation happened (for example, for endangered Mediterranean moufflon), but the offspring never faced the adulthood [30]. Somatic cell nuclear transfer remains a prolonged and tedious process with remarkable insufficiency and high risk of failure at every of its stages. In the case of bucardo brought above, assuming that it was the successful one, the success/failure ratio would be as low as 0,1% [3j].

CRISPR/Cas9 and genome editing

In 2012, a mini – conference at Wyss Institute, Cambridge, took place, during which a world famous genetic engineer, professor George Church, presented a hypothetical way of de-extinction, in which the step of assembling the whole extinct genomes is skipped and the genomes of closest living relatives are instead used as templates. All that it takes is to

precisely indicate the spots in the genomes which are responsible for the differences between both species' characteristics and change exactly those ones [3k]. Obtaining the cells, in which the inserted genes are being expressed, would be the equivalent of taking tissue samples from an animal and the following steps would be just identical as in SCNT method. The genes of extinct individuals are active *in vivo* – what ensures about that is the result of an experiment carried out in 2008, in which the transcription - enhancing element of *Col2A1* Thylacine gene managed to express the reporter genes in mice embryos [31, Fig.3]. The key genes which determine the specific features of extinct animals' phenotypes are being searched for and investigated, for example a *TRPV3* gene in mammoths, which has a pleiotropic effect on thermoregulation, hair growth and fatty tissue distribution [32]. The mammoth haemoglobin molecule as well as its gene are also being analysed. It has been proven, for instance, that the oxygen affinity of mammoth haemoglobin is less temperature – dependent than the same affinity of the same molecule in Asian elephant [33].

The methods of genome editing are based on repair mechanisms, self-induced in broken DNA. Starting from the double-stranded break (DBS), they induce one of the following ways of repair: HDR – homology derived repair - and NHEJ – non-homologous end joining [34]. When it comes to de-extinction, the former may come useful: normally, the strand from homologous chromosome serves as a template for repair, but in de-extinction it would be an exogenous aDNA inserted to the cell [3l]. The tools, thought to enable us to make it all happen would be restriction enzymes, the most popular of which are used in the following systems: zinc-finger nucleases (ZFNs), TALENs (transcription activator-like effector nucleases) and the most recent of them – CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated) [34]. First two have successfully been used among others in medicine: thank to zinc-finger nucleases, the gene for chemokine receptor CCR5 was deleted, thus providing the HIV-infection-resistant cell line [35]. However, enzymes working in both the first and the second system mentioned have their limitations, for example the size of the molecule or the number of spots available to be repaired [34]. Thus, a turn to CRISPR/Cas9 system is seemingly a better option. It is an effect of co-evolution between bacteria and the fags and is used by the former to get rid of pieces of foreign DNA inserted into their genomes [36]. Here is how CRISPS/Cas9 works: the genetic material of pathogen is being cleaved into fragments (so-called protospacer sequences) and inserted into bacterial genome. Those sequences are then transcribed as crRNA (CRISPR-RNA), which with bacterial nuclease Cas9 and trans-activating RNA (tracrRNA) altogether compose a complex, which binds foreign genome. Nuclease, the same in different bacteria species, binds to PAM – protospacer adjacent motif, and both strands are cut 3 base pairs above the PAM sequence [34]. On the upsides of the system there is, among others, the ability to change more than one locus in the genome at one time and the ease of programming the enzymes [34, 36]. Nevertheless, the risk of an adverse mutagenesis is still there, and as an example of diminishing it, the protospacer RNA is shortened [37]. For the time being, professor Church's team has managed to obtain elephant cells, in which 14 genes are replaced by their mammoth versions [38]. The CRISPR/Cas9 system is renowned for being successfully applied to both agriculture and medicine [39, 40].

Primordial germ cells

The de-extinction of birds poses a challenge for scientists. Due to their breeding specificity, their gametes cannot undergo genetic manipulations. Therefore, scientists aim at primordial germ cells (PGCs), which are carried with bloodstream to, at that time yet slightly developed,

embryo's gonads. All this takes place within the first 24 hours after the egg has been laid. Birds developed from theses embryos (the first generation) would maintain their species' phenotypes, as only their gametes would include inserted genes. It is the second generation of birds, which would bear resemblance to the extinct ancestors. What makes the process easier is the fact, that PGCs, unlike somatic cells, need no re-programming. The idea stated above will probably be used in passenger pigeon (*Ectopistes migratorius*) revival plan [3m, Fig.4]. What is more, the mitochondrial genome of passenger pigeon has been assembled [41]. PGCs can grow *in vitro* and intercellular communication pathways among them are being further investigated [42]. With the use of new transposon system called *piggyBac*, the chicken cells with long-term GFP (green fluorescent protein) expression have been obtained, which makes grounds for more efficient transgenic cells production [43]. On the downside, only a small percentage of the cells is obtained *in vivo* from living individuals, thus the embryos in which cell migration could be insufficient, receive a 'boost', again with the help of *piggyBac* [3n, 44].

Conclusion

The advents of new technologies and the novelties in science have definitely advanced the progress towards de-extinction, but there is still open question remaining, whether or not it should take place. Tasks successfully completed at molecular and cellular level induce a wide range of questions about the future prospects on living creatures' lives. Interfering with nature and evolution evokes growing concerns itself, let alone the animals: finding a surrogate mother, their development and growth, their impact on existing ecosystems [26,4].

Probably one of the best application of de-extinction methods could be to use them in order to help the extinction-endangered living species. For example, if mammoth genes were inserted into Asian elephants' genomes, their chances to survive in cooler climates would increase [38]. Also, the therapeutic application is possible (in primary immunodeficiency syndromes or in screening) [34, 36].

De-extinction requires a further technology advance, which would make it more obtainable, as well as a deep consideration in the terms of ethics, society, finances and environment. According to these, it is still a vision remote in time.

CHARACTERISTIC	AUROCHS	THE HECK CATTLE
HEIGHT AT THE WITHERS	MALES: 1,7 m (on average) FEMALES: 1,5 m (on average)	MALES: 1,42 m (on average) FEMALES: 1,31 m (on average)
COAT	MALES: light brown to black , a fair strip along the back FEMALES: reddish brown, rarely black CALVES: reddish, rarely black difference in coat colouring between the sexes was significant	MALES: from black-spotted to red, brown, gray, black FEMALES: light brown, red, gray, black CALVES: reddish-brown difference in coat colouring between the sexes usually not remarkable
HORNS	typical, relatively long and thin, curved onward and inward	slightly curved, relatively thin and short, curved significantly upward, often lyra-shaped
OVERALL BODY BUILD	similar to square (limbs more or less of equal length as the trunk), significant difference between the volumes of front and hind body parts	because of relatively short limbs, the trunk seems longish, the overall body plan resembles a rectangle, little difference between the volumes of front and hind body parts
HEAD	relatively long and narrow	relatively short and wide
UDDER	small, hardly visible	variated, both the big and small ones are seen

Tab. 1 – The comparison of the characteristics of the aurochs (*Bos taurus primigenius*) and the Heck cattle.Based upon: *Table 1. Physical characteristics of the aurochs as compared with those of Heck cattle* in Vuure, T. van (2002). History, morphology and ecology of the Aurochs (*Bos taurus primigenius*). Lutra 45(1): 3-17. Used with the permission of the Author.

Fig. 1 – Jemyma and the foal TJ13 – property and the copyrights belong to The Quagga Association Project, published with the permission of the Project Co-ordinator, Sir Craig Lardner.

Fig. 2 – A mammoth calf – the reconstruction (property and copyright belong to Natural History Museum of Vienna; photography taken by the author personally, published with the permission of Naturhistorisches Museum Wien. In none of the remaining mammoth fossils enough hair to prepare such a reconstruction was preserved. In the picture above, the hair used originally belongs to Scottish Highland Cattle.

Fig. 3 – A thylacine (*Thylacinus cenocephalus*) – stuffed animal exhibit, property and copyright belong to Natural History Museum of Vienna; photography taken by the author personally, published with the permission of Naturhistorisches Museum Wien.

Fig.4 – A passenger pigeon (*Ectopistes migratorius*) - stuffed animal exhibit, property and copyright belong to Natural History Museum of Vienna; photography taken by the author personally, published with the permission of Naturhistorisches Museum Wien.

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