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ABSTRACT

The zona pellucida (ZP) is an extracellular envelope that surrounds mammalian oocytes. This coat participates in the interaction between gametes, induction of the acrosome reaction, block to polyspermy and protection of the oviductal embryo. Previous studies suggested that carnivore ZP was formed by three glycoproteins (ZP2, ZP3 and ZP4), being *ZP1* a pseudogene. However, a recent study showed the expression of four proteins in the cat.

In this study, *in silico* and molecular analyses were performed in several carnivores to clarify the ZP composition in this order of mammals. The *in silico* analysis demonstrated the presence of the *ZP1* gene in five carnivores: cheetah, panda, polar bear, tiger and walrus, whilst in the Antarctic fur seal and the Weddell seal there are evidences of pseudogenization. The molecular analysis showed the presence of four ZP transcripts in ferret ovaries (*ZP1*, *ZP2*, *ZP3* and *ZP4*) and three in fox ovaries (*ZP2*, *ZP3* and *ZP4*). The analysis of fox *ZP1* gene showed the presence of a stop codon.

The results of this study strongly suggest that four ZP genes are expressed in most carnivores, whilst *ZP1* pseudogenization has affected independently three families (Canidae, Otariidae and Phocidae) of the carnivore tree.

INTRODUCTION

The zona pellucida (ZP) is a translucent, glycoproteic and acellular matrix that surrounds mammalian oocytes. Other vertebrates have a similar structure, which is called the vitelline envelope in amphibians, chorion in fishes and the perivitelline envelope in birds (Wassarman, 1988; Tian *et al*. 1997; Hyllner *et al*. 2001; Sasanami *et al*. 2002; Monné and Jovine, 2011). The ZP functions are related with important events in oocyte formation and different steps during fertilization; being involved in folliculogenesis, the organization and differentiation of granulosa cells, recognition and binding to spermatozoa, the induction of the acrosome reaction (AR), the block to polyspermy and the protection of the oocyte and the oviductal embryo (Modliński 1970; Bleil and Wassarman 1980b; Florman and Storey 1982; Berger *et al.* 1989; Liu *et al.* 1996; Rankin *et al.* 1996, 1999, 2001; Benoff 1997; Fazeli *et al.* 1997; Dean 2004; Gupta and Bhandari 2011; Gupta *et al*. 2012; Tanihara *et al*. 2013; Cao *et al*. 2016).

Recently, the genome availability in some species and the development of different techniques; such as mass spectrometry, have allowed to study in depth the ZP composition in different species. The number of the analyzed species is growing fast; however, the information in carnivores is still scarce.

According to its ZP composition, placental mammals can be classified into three different categories. 1) species with a ZP formed by three proteins ZP1, ZP2 and ZP3, where *ZP4* is a pseudogene (to date, only the house mouse) (Bleil and Wassarman 1980a; Lefièvre *et al*. 2004; Evsikov *et al*. 2006; Goudet *et al*. 2008); 2) species showing three proteins, where *ZP1* is a pseudogene (pig, cow, dog, common marmoset, dolphin and tarsier) (Hedrick and Wardrip 1987; Noguchi *et al*. 1994; Goudet *et al*. 2008; Stetson *et al*. 2012) and 3) species with four proteins (ZP1, ZP2, ZP3 and ZP4), for instance, cat, hamster, human, rabbit, and rat (Hughes and Barratt, 1999; Lefièvre *et al*. 2004; Hoodbhoy *et al*. 2005; Izquierdo-Rico *et al*. 2009; Jiménez-Movilla *et al*. 2009; Stetson *et al*. 2012, 2015).

Carnivores are classified in two suborders (Caniformia and Feliformia); the divergence between these two suborders is dated around 60-65 Ma (Nyakatura and Bininda-Emonds, 2012; Zhang *et al*. 2013). Caniformia comprises 9 families: Canidae (foxes, wolves, dogs), Odobenidae (walrus), Otariidae (sea wolves), Phocidae (seals), Mephitidae (polecats), Procyonidae (racoons, coatis), Mustelidae (weasels, otters), Ailuridae (red panda) and Ursidae (bears), whereas Feliformia comprises 6 families: Hyaenidae (hyenas), Eupleridae (fossa, mongooses), Herpestidae (mongooses), Viverridae (genets, civets, binturong) Prionodontidae (linsangs) and Felidae (cats) whilst the family Nandiniidae (African palm civet) is a sister of the feliformians (Agnarsson *et al*. 2010; Nyakatura and Bininda-Emonds, 2012).

Focusing on the ZP composition in carnivores, previous studies have reported a *ZP1* pseudogenization in the dog with three stop codons at positions 151 (exon 3), 279 (exon 5) and 421 (exon 8) (Goudet *et al.* 2008). This finding lead some authors to believe that *ZP1* was a pseudogene in other carnivores, such as the cat. In fact, the presence of ZP1 had never been reported in a carnivore until 2015 when our research group described this protein in the cat zona pellucida by molecular and proteomic analyses (Stetson *et al.* 2015).

In GenBank database there are sequences submitted from five species: the cat (*Felis catus*): where sequences from the 4 glycoproteins have been submitted; the dog (*Canis lupus familiaris*): with completed sequences from ZP2 and ZP3 and a partial sequence of ZP4; the ferret (*Mustela putorius furo*): with only ZP3 submitted; the fox (*Vulpes vulpes*): with ZP2 and ZP3 sequences available and the stoat (*Mustela erminea*): with a partial sequence of ZP2 and complete sequences of ZP3 and ZP4 (see Table 1).

Thus, taking into account the bibliography available until now, two different ZP models are possible in carnivores: 1) carnivores with 4 proteins in their ZP: ZP1, ZP2, ZP3 and ZP4, such us the cat (Stetson *et al.* 2015); and 2) carnivores with 3 proteins in their ZP: ZP2, ZP3 and ZP4, being *ZP1* a pseudogen, like the dog (Goudet *et al.* 2008). For that reason, the aim of this study was to decipher the ZP composition in other carnivore families. *In silico* analyses were performed in the species were the genome is available (Antartic fur seal, cheetah, ferret, panda, polar bear, tiger, walrus and Weddell seal) and a gene expression analysis using RT-PCR was performed from ferret and fox ovaries; this allowed us to covered 8 different families of the carnivore tree (see Fig. 1).

MATERIALS AND METHODS

***In silico* analyses**

Phylogenetic analysis of *ZP1*

ZP1 sequences from seven species: cheetah (*Acinonyx jubatus*), ferret (*Mustela putorius furo*), panda (*Ailuropoda melanoleuca*), polar bear (*Ursus maritimus*), tiger (*Panthera tigris altaica*), walrus (*Odobenus rosmarus divergens*) and Weddell seal (*Leptonychotes weddellii*) were retrieved from GenBank and/or Ensembl databases (Table 2). All these predictions were checked manually to detect annotation errors especially close to splicing sites. Similarity searches were performed using BLAST and BLAT against the assembled genome of dog (*Canis lupus familiaris*) in ENSEMBL (http://ensembl.org) and against the draft genome assembly of Antarctic fur seal (*Arctocephalus gazella*) recently published by Humble *et al.* 2016 downloaded from Dryad (doi:10.5061/dryad.8kn8c). The sequence obtained was aligned to the other carnivoran sequences with Muscle implemented in Seaview (Gouy *et al*. 2010) and verified visually to predict exons missing from the ENSEMBL predictions. Only the exonic portions were kept for the phylogenetic analysis. It was also checked that the new sequence corresponded to a syntenic region of the corresponding chromosome. We added to the alignment our partial sequences of fox (*Vulpes vulpes*) and sequences of 2 species each of Perissodactyla (*Ceratotherium simum simum* and *Equus caballus)* and Chiroptera (*Pteropus alecto* and *Miniopterus natalensis*) that were used as outgroups. The appropriate model of evolution (GTR+G) was determined using Akaike information criterion (AIC) and jModeltest software (Posada and Crandall, 1998). Phylogenetic trees were reconstructed using maximum likelihood with PhymL

(Guindon *et al*. 2010) and the robustness of the nodes was estimated with bootstrap support (n=1000).

Bioinformatic analysis

Sequences were analyzed to determine the degree of similarity with other known sequences using “BLAST program” (Basic Local Alignment Search Tool) (http://www.ncbi.nlm.nih.gov/blast/). Direct comparison between two sequences was made with “ALIGN program”, and the multiple sequence alignment were carried out using “Clustal Omega” (http://www.ebi.ac.uk/Tools/msa/clustalo/).

The amino acid sequences were analyzed to predict the signal peptide and different domains with the following software packages: “signalP” (www.cbs.dtu.dk/services/SignalP/) and “smart genome” (www.smart.embl-heidelberg.de). The programs "NetOGlyc" (www.cbs.dtu.dk/services/NetOGlyc) and "NetNglyc" (www.cbs.dtu.dk/services/NetNGlyc) were used to predict potential O-linked and N-linked glycosylation sites, respectively. The theoretical protein molecular weight and mature protein molecular weight were calculated with “PeptideMass” from “ExPASy” (http://web.expasy.org/peptide_mass/).

<u>**Molecular analyses**</u>

Ovaries collection

Ferret ovaries were obtained from two females subjected to ovariectomy in “Veterinary Clinic Huellas” (Murcia, Spain); dog ovaries from two females were donated from “Veterinary Clinic La Alcayna” (Murcia, Spain) and ovaries from three females were donated by the Laboratory for Rabies and Wildlife (Nancy, France). The ovaries were immediately immersed in RNAlater (Sigma-Aldrich, USA) and kept at -80°C until use.

DNA isolation

Total DNA was extracted from ovaries of two foxes and two bitches using a QIAamp DNA Mini Kit (Qiagen, Germany) following manufacturer's recommendations.

Purification of ovarian RNA, cDNA synthesis and polymerase chain reaction amplification

Total RNA was isolated from ovaries of two ferrets, two foxes and two bitches using RNAqueous® kit (Ambion, USA) according to the manufacturer's instructions. The first-strand cDNA was synthesized with the SuperScript First-Strand Synthesis System kit for RT-PCR (Invitrogen-Life Technologies, USA), according to the supplier's protocol.

Ferret and fox ZP genes (*ZP1*, *ZP2*, *ZP3* and *ZP4*) and dog *ZP1* gene were amplified using polymerase chain reaction (PCR) by means of specific primers. Ferret primers were designed according to the cDNA sequences obtained from GenBank and Ensembl databases with the following accession numbers: ENSMPUT00000014555 for *ZP1*, XM_004780136 for *ZP2*, NM_001310185 for *ZP*3 and XM_004774783 for *ZP4*; AF038150 for *β-actin* was used as positive control.

ZP2 and *ZP3* fox primers were designed from the sequences of this species, which are available at the GenBank database with accession numbers AY598031 and

AY598032 respectively. To amplify *ZP1* and *ZP4*, primers were designed according to the predicted cDNA sequences of the dog, its closest relative in the GenBank database with accession numbers XM_014120952 for *ZP1* and XM_536329 for *ZP4*. To amplify dog *ZP1*, primers were designed according to the predicted sequence XM_014120952.

PCR amplifications were performed using 2 µl of target cDNA, 0.5 µg of each primer, 200 µM of each dNTP, and 1 IU of Taq DNA Polymerase (Fermentas, USA). PCR was carried out using an initial denaturation cycle of 3 min at 95 ºC, and then 30 cycles of 1 min at 95ºC, 1 min at annealing temperature (depending on the primers) and then 1 min at 72ºC. The final extension time was 10 min at 72ºC. PCR products were analyzed by electrophoresis on 1.5 % agarose gels; four microliters of the PCR reaction mixture were mixed with loading buffer (Fermentas, USA) and separated for 60 min at 90 V. The gel was immersed in ethidium bromide (Sigma-Aldrich, USA) and visualized under UV light.

When multiple bands were obtained, amplicons of the expected size were carefully excised from the agarose gels and purified with the QIAquick Gel Extraction Kit Protocol (Quiagen, Germany) according to the manufacturer's protocol. When only one band was obtained, it was directly purified with the DNA Clean & ConcentratorTM-5 (Zymo, USA) according to the kit instructions. After that, the amplicons were automatically sequenced using 3500 Genetic Analyzer (Applied Biosystem, USA).

Amplification of the exon 8 of *ZP1* in fox and dog

Considering that three stop codons were described in exons 3, 5 and 8 of dog ZP1 inducing the pseudogenization (Goudet *et al*. 2008); exon 8 was amplified in the fox. Furthermore, as a control of our results the same experiment was conducted in the dog. Primers and the conditions for the PCR amplification were identical to that used in the fox cDNA (as explained in the previous section).

Amplifications by PCR were performed using 100 ng of target DNA, 0.5 μg of each primer, 200 μM of each dNTP, and 1 IU of Taq DNA Polymerase (Fermentas, USA). PCR amplifications, sample purification and sequencing were carried out as explained above.

RESULTS

***In silico* analysis**

Taking into account that *ZP1* is a pseudogen in the dog (Goudet *et al*. 2008) this event could have affected other species of carnivores. To explore this possibility an *in silico* analysis was made using the *ZP1* sequences from Antartic fur seal, cheetah, tiger, ferret, panda, polar bear, walrus and Weddell Seal corresponding to 6 different families (Otariidae, Felidae, Mustelidae, Ursidae, Odobenidae and Phocidae) which are available in GenBank, Ensembl or Dryad databases. The ORF of *ZP1* from six of these species, have an initial ATG and a terminal stop codon, not showing evidences of pseudogenization. The alignment among the species shows a high degree of similarity, presenting the typical architecture of the ZP proteins, with a signal peptide, a trefoil domain (present in ZP1 and ZP4 proteins), the ZP domain, a furin cleavage-site (Arg-Gln-Arg-Arg) conserved in all the species and the transmembrane domain (Fig. 2). In the different species analyzed there are two putative N-glycosylation sites; Asn75, which is conserved in the seven species aligned, and Asn362, conserved in all of them, with the exception of the polar bear. The comparative analysis of this sequence revealed high similarity with ZP1 from other species. The amino acid sequence of ferret ZP1 is 79% identical to walrus ZP1, 78% to tiger, 76% to cheetah, cat and panda and 75% to polar bear.

On the other hand, the genome analysis made in the Antarctic fur seal and the Weddell seal showed evidences of pseudogenization. In the Antarctic fur seal, there are two indels present in exon 3 and exon 4 that lead to the presence of stop codons. In the Weddell seal, the initial ATG is replaced by ACG, furthermore, it revealed the presence of different indels: two deletions in exon 3, one deletion in exon 8 and one deletion in exon 12, and the insertion of a "G" in exon 4 and a "T" in exon 12. These indels of 1 base pair disrupt the open reading frame of the gene and lead to the presence of several stop codons. Moreover, a mutation at the splicing site at the end of exons 7 and 9 was found (Fig. 3).

Molecular analyses

Ferret *ZP1*, *ZP2*, *ZP3* and *ZP4* mRNA amplification

Ferret *ZP1* mRNA was totally amplified. Furthermore, partial amplifications of *ZP2*, *ZP3* and *ZP4* mRNA were made to confirm the presence of four transcripts in ferret ovaries.

ZP1 mRNA contains an ORF of 1878 nucleotides. This sequence was submitted to GenBank database with accession number KX583606. The ATG initiation codon predicted with the Pedersen and Nielsen algorithm (Pedersen and Nielsen, 1997) was found to be associated with vertebrate initiator codons (Kozak, 1991). The sequence contains a stop codon (TAA) in positions 1879-1881. The ORF of ZP1 codifies for a polypeptide 626 amino acids long with a theoretical molecular weight of 67.34 kDa. The sequence contains a signal peptide of 24 aminoacids long between Thr24 and Gln25, predicted by the Bendtsen algorithm (Bendtsen *et al.* 2004) and a furine cleavage site in Gln537 (Duckert *et al.* 2004), being 55.79 kDa the expected molecular weight of the mature protein (Fig. 4).

This ZP protein shares domains with other proteins of the same family, the archetypal 'ZP domain', a signature domain comprising 272 amino acid residues (Gln262-Gly533) with ten Cys residues. The trefoil domain, characteristic of ZP1 and ZP4 is also present; this part contains 44 residues (Glu217-Thr260) with 6 Cys residues. The last domain is the transmembrane domain (TMD) between Leu586 and Leu608 with 23 amino acids; followed by a cytoplasmic tail. A basic amino acid domain (Arg536-Gln-Arg-Arg539) upstream of the TMD may serve as a consensus furin cleavage site (Fig. 4) (Boja *et al.* 2003, 2005; Duckert *et al.* 2004).

A total of 89 potential O-glycosylation sites were predicted in the ZP4 protein and two potential N-glycosylation sites (Asn-X-Ser/Thr) are present in the mature protein at the position (Asn75 and Asn362) (Fig. 4).

Fox *ZP1*, *ZP2*, *ZP3* and *ZP4* mRNA amplification

The mRNA of fox *ZP4* was totally amplified. Although the ORFs corresponding to fox ZP2 and ZP3 were previously characterized (Harris *et al*. 1994; Okazaki *et al*. 1995; Okazaki and Sugimoto, 1995), partial amplifications of each transcript were made to confirm the above results (Fig. 5).

Full-length fox *ZP4* mRNA contains an ORF of 1704 nucleotides; containing a stop codon (TAG) in positions 1705-1707. This sequence was submitted to GenBank database with accession number KF956365. The ORF of ZP4 codifies for a polypeptide 568 amino acids long with a theoretical molecular weight of 63.26 kDa. The signal peptide is 16 aminoacids long between Ala16 and Leu17, which was predicted by the Bendtsen algorithm (Bendtsen *et al*. 2004) and a furin cleavage site in Gln499 (Duckert *et al*. 2004). The expected molecular weight of the mature protein is 53.87 kDa (Fig. 6).

The ZP domain comprises 274 amino acid residues (His224-Ala497) with ten Cys residues. The trefoil domain contains 45 residues (Asp178-Thr222) with 6 Cys residues; and the transmembrane domain (TMD) between Thr544 and Ile566 is 23 amino acids long; is followed by a cytoplasmic tail. A basic amino acid domain (Arg498-Gln-Arg-Arg501) upstream of the TMD may serve as a consensus furin cleavage site (Fig. 6) (Boja *et al*. 2003, 2005; Duckert *et al*. 2004).

A total of 89 potential O-glycosylation sites were predicted in the mature protein and two potential N-glycosylation sites (Asn-X-Ser/Thr) are present in the mature protein at the positions Asn44 and Asn68 respectively (Fig. 6).

On the other hand, the presence of *ZP1* mRNA in fox ovary was studied in this work, using PCR amplifications from cDNA. However, none of them had success. Thus, parallel experiments using cDNA and gDNA were conducted using the same primers. An amplicon of *ZP1* was obtained only from gDNA (see Fig. 5), suggesting that there is not expression of this gene in fox ovaries. Furthermore, as a control, same amplifications, with identic primers were conducted in the dog using cDNA and gDNA, like in the fox a PCR amplification was only obtained when gDNA was used.

Considering that Goudet *et al*. in 2008 described the presence of three stop codons in the exons 3, 5 and 8 of dog ZP1, an amplification of exon 8 was performed in both species (fox and dog). The sequences shown a high degree of similarity between both sequences and the presence of a stop codon conserved in both of them (Fig. 7). This event could be the reason of the pseudogenization in fox *ZP1*, not allowing the expression of the mRNA in its ovaries.

Phylogenetic analysis

The phylogenetic tree reconstructed with all the *ZP1* sequences found in databases and with our new ones is presented in figure 8. The phylogeny of ZP1 is congruent with phylogenies obtained in previous studies (e.g. (Nyakatura and Bininda-Emonds, 2012) (Fig.1). Four species in the phylogeny presented in this work are affected by the pseudogenization, the dog and the fox, both belonging to the Canidae family, the Antartic fur seal, belonging to the Otariidae family and the Weddell seal, belonging to the Phocidae family. Whereas, the walrus, belonging to the family Odobenidae, does not present evidences of pseudogenization. These results indicate that there was at least three pseudogenisation events in the Carnivora order, one that took place in the lineage leading to dogs and foxes, a second one in the lineage leading to the Weddell seal and the third one in the lineage leading to the Antarctic fur seal (Fig. 8).

DISCUSSION

Zona pellucida composition in carnivores

The ZP is an extracellular matrix surrounding the oocyte and the early embryo and it is involved in important steps during fertilization. The ZP composition is different among the species, being formed by 3 or 4 proteins. The differences in composition are mainly due to a pseudogenization process or death of genes (Goudet *et al*. 2008)**.** Previous studies showed that the ZP pseudogenization has affected different mammalian orders, such us the pseudogenization of *ZP4* in the house mouse (Bleil and Wassarman 1980a; Lefièvre *et al*. 2004; Evsikov *et al*. 2006); or the pseudogenization of *ZP1* in the pig, cow, dog, common marmoset, dolphin and tarsier (Hedrick and Wardrip 1987; Noguchi *et al*. 1994; Goudet *et al*. 2008; Stetson *et al*. 2012). In the case of carnivores, the pseudogenization of *ZP1* was expected, at least in Caniformia, according to the data described in the dog (Goudet *et al*. 2008).

Different authors have submitted sequences from ZP2, ZP3 and ZP4 corresponding to different species; for instance the stoat (Jackson and Beaton, 2004), ferret (Jackson and Beaton, 2004), dog (Harris *et al*. 1994; Okazaki *et al*. 1995; Okazaki and Sugimoto, 1995; Srivastava *et al*. 2002; Blackmore *et al*. 2004; McLaughlin *et al*. 2004) and fox (Beaton and Bradley, 2004; Reubel *et al*. 2005), but as far as we are concern ZP1 was never reported in those species (see table 1). In the cat, the protein sequences submitted to the gene database until 2013, like in the rest of carnivores were ZP2, ZP3 and ZP4 (Harris *et al*. 1994, 1995; Okazaki and Sugimoto, 1995; Jewgenow and Fickel, 1999; Okazaki *et al*. 2007; Eade *et al*. 2009); however in 2015, our group confirmed the presence of ZP1 on this species by means of molecular and proteomic analyses, being the first carnivore with 4 ZP proteins described (Stetson *et al*. 2015). Thus, we considered that the reanalysis of the ZP composition in this group of mammals was necessary to shed light on the carnivore ZP composition.

Zona pellucida pseudogenization in carnivores

Carnivores are classified in two suborders (Caniformia and Feliformia); thus, the presence of a functional ZP1 in the cat indicated that the pseudogenization could have affected only the suborder Caniformia; after the divergence between these two suborders, event dated around 60-65 Ma (Nyakatura and Bininda-Emonds, 2012; Zhang *et al*. 2013). This hypothesis is reinforced in this work by the fact that a functional *ZP1* gene was evidenced in tiger and cheetah, two other Feliformia species (Fig. 1).

The analysis of the genomic data of several species of Caniformia led us to discover that ferret, panda, polar bear and walrus present a sequence corresponding to *ZP1*, which seems to be functional. However, different events in the sequence of the Antarctic fur seal and the Weddell seal indicate that *ZP1* is not a functional gene in these species; suggesting that *ZP1* pseudogenization occurred in the Otariidae and Phocidae families. Nevertheless, none of these events were found in the walrus (Odobenidae family), all of them belong to the same superfamily Pinnipedia; thus, it would indicate that the pseudogenization of the *ZP1* gene in the lineage of the Antarctic fur seal and the Weddell seal was produced after the separation of these two families, event estimated around 22 Ma (Nyakatura and Bininda-Emonds, 2012). As there are more indels and defective mutations in the Weddell seal than in the Antarctic fur seal

ZP1 sequence, we could hypothesized that the pseudogenisation is more ancient in the Weddell seal lineage.

Additional sequence data of other Otariidae and Phocidae would be necessary in order to determine more precisely when the pseudogenisation took place.

Apart from the *in silico* analyses, the presence of the mRNA codifying for the different ZP proteins has been explored in two species; the ferret and the fox. These species were chosen due to its interest to control their populations. In ferret, only the protein ZP3 was previously described (Jackson and Beaton, 2004). In this work, *ZP1* was totally amplified and fragments from *ZP2*, *ZP3* and *ZP4* genes were amplified and automatically sequenced, demonstrating the presence of four transcripts in ferret's ovaries.

Considering the results obtained in ferret, the expression of *ZP1* was explored in the fox too. In this species, only ZP3 protein was previously described (Reubel *et al*. 2005). In this study, the mRNA of different ZPs was amplified from fox ovaries. The open reading frame of red fox *ZP4*, present in all carnivores studied until this date, was completely amplified, moreover partial sequences of the open reading frame of *ZP2* and *ZP3* were also obtained. To ascertain whether *ZP1* pseudogenization also affected the foxes, the exon 8 of dog *ZP1* was amplified in dog and fox, as a stop codon was previously described in the dog (Goudet *et al*. 2008). Our results indicated that this stop codon was conserved in both species. These results confirm that the *ZP1* pseudogenization also affected the fox branch. Thus, this event probably occurred after the separation of the Canidae from the other carnivorous families and as this event affected both subfamilies (Canini and Vulpini), it probably took place between 15-60 Ma (Nyakatura and Bininda-Emonds, 2012).

These results indicate that, the pseudogenization of *ZP1* has been produced several times along mammalian evolution, affecting other orders than Carnivora. For instance, it was documented also in the cow (Goudet *et al*. 2008), common marmoset, dolphin, pig and tarsier (Stetson *et al*. 2012). In this work, the pseudogenization of *ZP1* has been reported in three more species, the fox, the Antarctic fur seal and the Weddell seal. On the other hand, other studies reported the pseudogenization of *ZP4* in mammals; for instance in the house mouse (Bleil and Wassarman 1980a; Lefièvre *et al*. 2004; Evsikov *et al*. 2006; Goudet *et al*. 2008); whereas, ZP2 and ZP3 proteins are present in all the species described to this date, meaning that the functions developed by these proteins are essential (Liu *et al*. 1996; Rankin *et al*. 1996, 2001; Dean, 2004; Goudet *et al*. 2008; Baibakov *et al*. 2012; Avella *et al*. 2014, 2016).

Thus, during the evolution of vertebrates the pseudogenization of *ZP1* or *ZP4* genes has been a common event. These two genes, come from the duplication of a common ancestral gene (Bausek *et al*. 2000; Goudet *et al*. 2008); some species maintain both copies (ZP1 and ZP4), whilst others only one (ZP1 or ZP4).

On the other hand, it is not the first time that a gene is loosed several times during the evolution affecting different evolutionary lineages; for instance, the pseudogenization of the olfactory receptor genes was previously reported in primates, coinciding with the acquisition of the trichromatic vision (Gilad *et al*. 2004). The pseudogenization of *ZP1* or *ZP4* in several lineages of mammals remains unclear, further investigations are needed to clarify this aspect.

Zona pellucida contraception

For years ZP proteins have been used to develop contraceptive vaccines in mammals; such us cats (for a review see Levy, 2011), dogs (for a review see Gupta *et al*. 2011; Maenhoudt *et al*. 2014), elephants (Delsink *et al*. 2007), feral horses (Joonè *et al*. 2015), kangaroos (Kitchener *et al*. 2009a), koalas (Kitchener *et al*. 2009b), white-tailed deers (Rutberg *et al*. 2013), etc. The description of *ZP1* in ferret could be important for developing contraceptive vaccines to control the population size of this species. This could be relevant in countries like New Zealand, where the number of these predators is out of control (McLennan JA *et al*. 1996; Wilson PR *et al*. 1998; Jackson RJ *et al*. 2007; D Prada *et al*. 2014). Vaccines with native ZP from mink and ferret were produced and tested in the cat; animals responded to immunization with an antibody production; however, no reactivity was observed after an immunohistochemistry analysis and all the cats were pregnant after a breeding trial (Levy *et al*. 2005). Nevertheless, as far as we are concern, none of these vaccines have been tested in the Mustelidae population.

A recent study revealed that a homozygote mutation in human ZP1 induces infertility in women (Huang *et al*. 2014) and it was demonstrated that ZP1 binds to the spermatozoa and induces the acrosome reaction in humans (Ganguly *et al*. 2010a, 2010b). Thus, considering that the ZP composition is similar in humans, ferrets and cats, the development of a contraceptive vaccine including ZP1 as an antigen could be beneficial to induce contraception in these carnivores.

Like in the ferret, several authors point out the need to control the fox population; as it is a predator of native and endangered species and a reservoir of zoonotic diseases, like the rabies (Bradley 1994; Robinson and Holland 1995; Artois 1997; Suppo *et al*. 2000; Smith and Wilkinson 2003). As far as we are concern, in the fox, only the recombinant porcine and fox ZP3 proteins have been tested to induce contraception, but no immune response was obtained with either of them (Reubel *et al*. 2005).

It should be considered that, previous studies demonstrated that the use of native porcine ZP to induce contraception in seals (grey seals, harp seals and hooded seals), produces a good and a long response to a single dose administration (Brown *et al*. 1997a, 1997b). Antarctic fur seals, Weddell seals and pigs present only three proteins in their ZP, with a high homology (71% for ZP2, 74% for ZP3, 68% and 70% for Weddell seal ZP4 isoforms X1 and X2 respectively); the fox presents the same composition model, thus it may be indicated to test these three proteins (porcine and fox) to induce contraception in the fox. However, the use of porcine ZP in bitches induces side effects (Mahi-Brown *et al*. 1982, 1985, 1988), whilst recent studies indicate that recombinant dog ZP3 could be a promising candidate to induce contraception in dogs (Gupta *et al*. 2011). Further studies are necessary to develop an efficient vaccine to control the wildlife population and for the management of street cats and dogs.

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Figure 1. Phylogeny and divergence-time estimates for the taxa involved in our study. For each taxon the family name is indicated within brackets. Horse and cow are used as outgroups. The ages of the nodes are from Nyakatura and Bininda-Edmonds, 2012.

Figure 2. Comparison of ZP1 amino acid sequences from tiger, cheetah, cat, panda, polar bear, ferret and walrus. The accession numbers of the sequences used are: tiger (XP_007092072), cheetah (XP_014930922), cat (AEI98737), panda (XP_002928701), polar bear (XP_008705767), ferret (KX583606) and walrus (XP_012421243). Identical amino acids are marked by an asterisk (*), colon (:)

represents conserved residues and dot (.) represents semi-conserved residues. The signal peptide is marked in pink. The trefoil domain is shown in blue. The zona domain is shown in red. The consensus furin cleavage-site is underlined. The transmembrane domain is marked in orange. The cystein residues are marked in green. The potential N-glycosylation sites are shown in purple.

Figure 3. Comparison of Antarctic fur seal, Weddell seal and walrus ZP1 sequences. The first ACG codon, the indels and the stop codons (*****), which are indicative of *ZP1* pseudogenization in the Antarctic fur seal and the Weddell seal are shown in bold on gray background.

Figure 4. Nucleotide and deduced amino acid sequence of ferret ZP1. The initial and final codons are in pink. The signal peptide is marked with green colour. Trefoil domain is shown in blue. The zona domain is shown in red. The consensus furin cleavage-site is underlined. The transmembrane domain is marked in orange. The putative N-glycosylation sites (Asn75 and Asn362) are indicated in violet. In capital letters and bold: two polymorphisms (positions: 885 (Y) and 1296 (Y)).

Figure 5. Analysis of *ZP1*, *ZP2*, *ZP3* and *ZP4* gene expression in fox ovary by PCR. Amplicons corresponding to each gene are shown. In line 1 a fragment of *ZP1* amplified from gDNA is shown. In line 2 *ZP1* was not amplified from cDNA. Lines 3, 4 and 5 show fragments of *ZP2*, *ZP3* and *ZP4* genes amplified from cDNA.

Figure 6. Nucleotide and deduced amino acid sequence of fox ZP4. The initial and final codons are in pink. The signal peptide is marked with green colour. Trefoil domain is shown in blue. The zona domain is shown in red. The consensus furin cleavage-site is underlined. The transmembrane domain is marked in orange. The putative N-glycosylation sites (Asn44 and Asn68) are indicated in violet.

Figure 7. Comparison of ZP1 amino acid sequences (exon 8) from dog and fox. Identical amino acids are marked by an asterisk (*) and colon (:) represents conserved residues. The stop codon in both species is signaled by an arrow (↓). This stop codon along with others stop codons or modifications could be responsible of the *ZP1* pseudogenization.

Figure 8. Phylogenetic relationship of the *ZP1* gene in carnivores. Bootstrap support is indicated for each node. Species with *ZP1* pseudogenised (dog, fox, Antarctic fur seal and Weddell seal) are indicated in gray background.

Figure 1

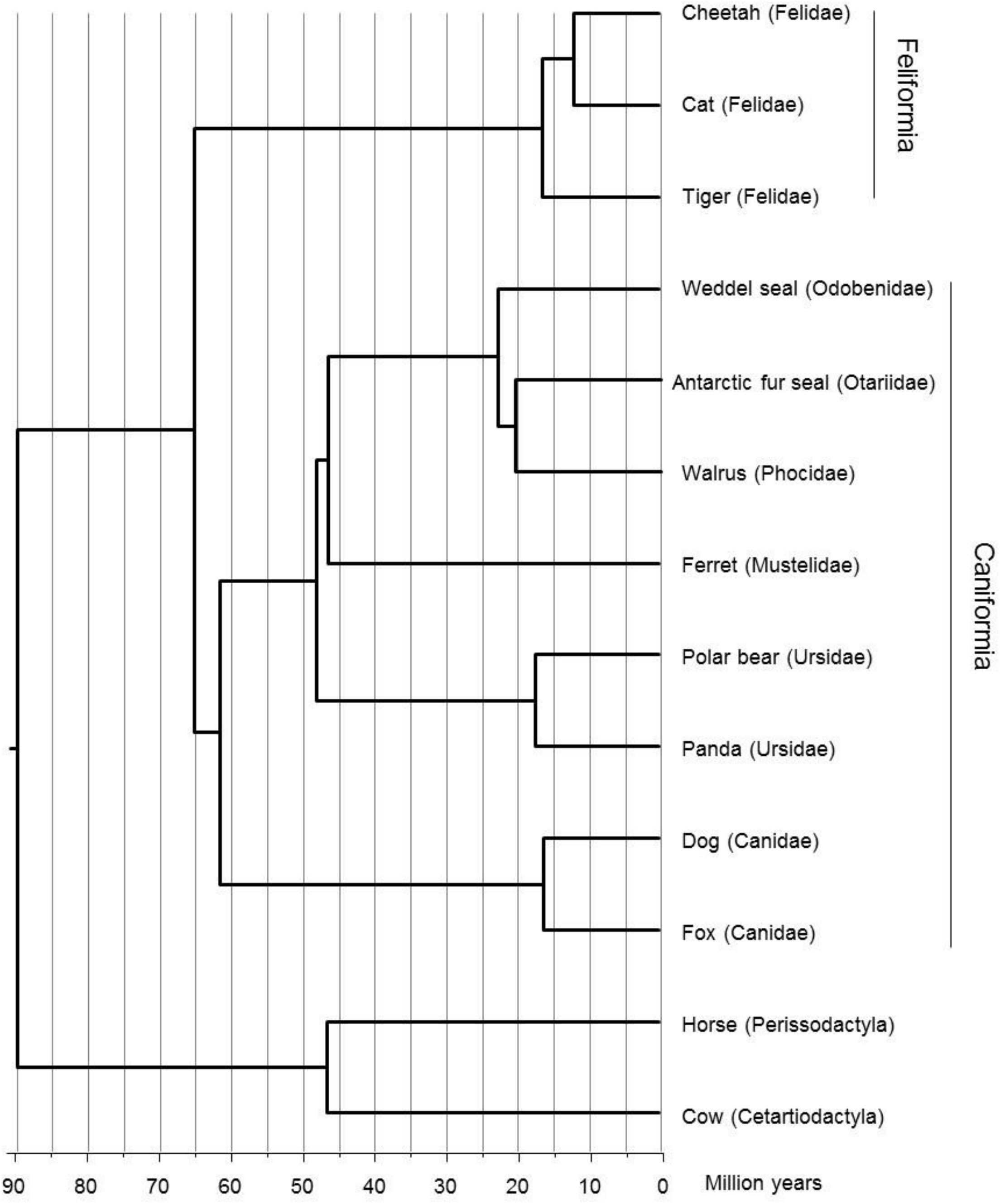
Cheetah (Felidae)
Cat (Felidae)
Tiger (Felidae)
Feliformia
Weddel seal (Odobenidae)
Antarctic fur seal (Otariidae)
Walrus (Phocidae)
Ferret (Mustelidae)
Polar bear (Ursidae)
Panda (Ursidae)
Dog (Canidae)
Fox (Canidae)
Caniformia
Horse (Perissodactyla)
Cow (Cetartiodactyla)
90
80
70
60
50
40
30
20
10
0
Million years

Figure 2

```
Tiger          MVAVVYLTTRALAMVWDGCVALLLLLLVAALGLGQRPHPEPGLRGLRHSSDCGIKGMQLL  60
Cat            MVAVVYLMTRASAMVWDGCVALLL-LLVAALGLGQRPHPEPGLRGLRHSSDCGIKGMQLL  59
Cheetah        MVAVVYLMTRASAMVWNGCLELLL-LLVAALGLGPQPHPEPGLRGLRHSSDCGIKGMQLL  59
Panda          -------MAGASARVWGCCVALL--LLLAALGLGQRPHPEPGLAGLWHRYDCGVKGMQLQ  51
Polar bear     ---MVCLMAGASAGVWCRCMALL---LLAALGLGQRPHPAPGLTGLWHHYDCGVKGMQLR  54
Ferret         -------MAGISARLRDGCVALL---LVAALGLTQRPHTEPGPSGLWHGYDCGVKGMQLW  50
Walrus         -------MAGASAGVWDCHVALL---LVTALGLGQRLHPKPGLSSLGYSYDCGVKGLQLR  50
                       :   * :    : **   *::****  : *  **  .* :  ***:**:**

Tiger          VFPRPGQTVRFKVVDEFGNQFEVHNCSVCYHWVTARPLGPAVFSADYRGCHVLEKDGRFH 120
Cat            VFPRPGQTVRFKVVDEFGNQFEVHNCSVCYHWVTARPLGPAVFSADYRGCHVLEKGGRFH 119
Cheetah        VFPRPGQTVRFKVVDEFGNQFEVHNCSVCYHWVTARPLGPAVFSADYRGCHVLEKDGRFH 119
Panda          VFPRPGQMIRFKVVDEFGNQFEVNNCSACYHWVTTKPLGPAVFSAGYKGCHVLEKDGRSH 111
Polar bear     VFPQPGQTIRFKVVDEFGNQFEVNNCSACYHWVTTKPLGPAVFSAGYKGCHVLEKDGRSH 114
Ferret         AFPGPGQTIRFKVVDEFGNQFEVNNCSACYHWVTTKPPGHAVFSAGYKGCHVLEKDGRSH 110
Walrus         VLPQSGQMVRFKVVDEFGNQFEVNNCSACYHWVSTKPQAPAVFSAGYKGCHMLEKDGRSH 110
               .:*  ** :**************.***.*****:::* . ***** *:***:*** ** *

Tiger          LRVFVEAVLRDGRVDAAGEVTLICPKPGHTWTPESHLASRTGFSLPTPHTRPLRPTREHS 180
Cat            LRVFVEAVLRDGRVDAAGEVTLICPKPGHTWTPESHLASRTGFSLPTPHTRPLRPTQEHS 179
Cheetah        LRVFVEAMLRDGRVDAAGEVTLICPKPGHTWTPESHLASRTGFSLPTPHTRPLRPTQEHS 179
Panda          LRVFIEVVLPDGRVDATRDVTLICPKPGHTWTPDTHLAPHTGFSLPTPQARPLHPTPERG 171
Polar bear     LRVFIEAVLPDGRVDATRDVTLICPKPGHTWTPDAHLAPHTGFSLPTPQARPLHPTPEHG 174
Ferret         LKVIIEAVLPNGQVEATGDVTLICPKPAHTWTPDPHLAPRTGFSRPTPQAWSLRPNPEHS 170
Walrus         LTVFIEAVGPDGRVDATRDVTLICPKPGHAWTPASRPEPPVGFSLPTPQARPLRPIPEHG 170
               * *::*.:  :*:*:*: :********.*:***  :    .*** ***::  *:*  *:.

Tiger          FTRPTPALLPLRPGA-TRPTLTPPPWDILEHWGVDEPLHPGAPLTWEQCQVPSGHIPCVV 239
Cat            FTRPTPALLPLRPGA-THPTLTLPQWDILEHWGVDEPLHPGAPLTWEQCQVPSGHIPCVV 238
Cheetah        FTRPTPALLPLRPGA-TRPTLTLPQWDILEHWGVDEPLHPGAPLTWEQCQVPSGHIPCVV 238
Panda          LVHATPTLLSLRPGPTTHPTQAPPQWGTLEHWGGSEPPYPGAHLPRERCQVPSGPIPCGV 231
Polar bear     LVRATPTLPSLRPGPTTHPTQAPPQWGTLEHWGGSEPPYPGAHLPREQCQVPSGPIPCGV 234
Ferret         FVHATPALPSLGPGPTSHATQAPPQGGTLRPWGVDEPPYSGAPLTPELCQVPSRAISCGV 230
Walrus         FVRATPALPSLEPGPTTHPTQAQPQWGTLEHGGVDKPPYPGMRLTPGRCQVSSRPIPCGV 230
               :.: **:*  * **  :: * : *    *.  * .:* : *  *    *** *  * * *

Tiger          RRGSKEACQKAGCCYDNRRGVPCYYGNTATVQCFRNGHFVLVVSQETALAHGITLANIHV 299
Cat            RRGSKEACQKAGCCYDNSRAVPCYYGNTATVQCFRNGHFVLVVSQETALAHGITLANIHV 298
Cheetah        RRGSKEACQKAGS----SSAVPCYYGNTATVQCFRNGHFVLVVSQETALAHGITLANIHV 294
Panda          RRGSKEACQRAGCCYDNSREVPCYYGNTATVQCFRNGHFVLVVSREIALAHGITLASIHL 291
Polar bear     RRGSKEACQRAGCCYDNSREVPCYYGNTATVQCFRNGHFVLVVSRETALAHGITLANIHL 294
Ferret         GRSSKEACQQAGCCYDNSRAIPCYYGNTATVQCFRNGHFVLVVSQETALAHGITLANLHM 290
Walrus         R-SSEEACLRAGCCYDNSREVPCYYGNTATVQCFRNGHFVLVVSRETALAHGITLANIHM 290
                 .*:*** :**.       :***********************:* *********.:*:

Tiger          AYAPTRCSPTQDTGSFVVFQFPLTHCGTTVQVVGNQLLYENQLVSDIDVQMGPQGSITRD 359
Cat            AYAPTSCSPTQDTGSFVVFQFPLTHCGTTVQVVGNQLLYENQLVSDIDVRMGPQGSITRD 358
Cheetah        AYAPTSCSPTQDTGSFVVFQFPLTHCGTTVQVVGNQLLYENQLVSDIDVRMGPQGSITRD 354
Panda          AYAPTSCSPTQETRSFVVFRFPFSHCGTTVQVAGDQLIYENQLVSDIEAQTGPQGSITRD 351
Polar bear     AYAPTSCSPTQETRSFVVFRFPFSHCGTTVQVAGNQLIYENQLVSEIEARTGPQGSITRD 354
Ferret         AYAPTGCSPTQETGSFVVFRFPLSHCGTTAQVAGNQLVYENQLVSDIEARTGPQGSITRD 350
Walrus         AYAPTSCSPAQKTGSFVVFRFPFSHCGTTVQVAGNQLIYENQLVSDIEAQTGPQGSITRD 350
               ***** ***:*.* *****:**::*****.**.*:**:*******:*:.: *********

Tiger          GTFRLHVR------CIVNASDFLPLRASIFPPPSPAPVIQSG------------PLRFQL 401
Cat            GAFRLHVR------CTVNASDFLPLQASIFSPPSPVPVIQSG------------PLRFQL 400
Cheetah        GAFRLHVR------CTINASDFLPLRASIFPRPSPAPVIQSG------------PLRFQL 396
Panda          GTFRLQAR------CVFNASDFLPLRASVSPRPSPAPVTQSXXXXXAPPPPPPSPPRLRA 405
Polar bear     GTFRTWLVPGPVSGCSTSSWSTRPRLESSLPEVKGEPLGAGGKG-------GWGQLSLQN 407
Ferret         GTFRLHMR------CIFNASDFLPLQASIFPPPSPAPVTQSG------------PLHLQL 392
Walrus         GTFRLHVR------CVFNTSDFLPLQASIFLPPSPAPVTQSG------------PLQLQL 392
               *:**          *  .: .  *   *     .  *:  .                ::

Tiger          RIATDETFRSFYEEGDYPIVRLLREPVSVEVRLLDRTDPGLVLLLHRCWATPSASPFQQP 461
Cat            RIATDETFRSFYEEGDYPIVRLLREPVSVEVRLLDRTDPGLVLLLHQCWATPGVSPFQQP 460
Cheetah        RIATDETFRSFYEEGDYPIVRLLREPVSVEVRLLDRTDPGLVLLLHRCWATPSVSPFQQP 456
Panda          PLPADETFRSFYGPGDYPIVRLLREPAAVEVRLLQRADPGLVLLLHQCWATPGASPFQQP 465
Polar bear     MGCRAWTFRSYYEAGDYPIVRLLPEPVPVEVRLLQRADPGLVLLLHQCWATPGASPFQQP 467
Ferret         RIAKDETFRSFYEEGDYPLVRLLREPVPVEVRLLHRTDPGLVLLLHQCWATPGASPFQQP 452
Walrus         RIAKDESFRSYYEEGDYPLVRLLRQPVPVEVRLLERTDPSLVLLLHQCWATPGANPFQQP 452
```

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                   :***:*  ****:**** :*. ******.*:**.******:*****...*****

Tiger        QWPILSEGCPFDGDSYRTRMVASDGAGLSFPSHHQRFTVTTFALLDPGSQRALRGQVYFF 521
Cat          QWPILSEGCPFDGDSYRTRMVASDGAGLSFPSHHQRFTVTTFALLDPDSQRALRGQVYFF 520
Cheetah      QWPILSEGCPFDGDSYRTRMVASDGAGLSFPSHHQRFTVTTFALLDPDSQRALRGQVYFF 516
Panda        QWPILSDGCPFDGDNYRTQLVALDGAGLSFPSHYQRFTVAVFALLDPGSRRALGGWVYFF 525
Polar bear   QWPILSDGCPFDGDNYRTQLVALDGAELSFPSHYRRFTVATFALLDPGSRRALGGWVYFF 527
Ferret       QWPILSEGCPFDGDSYRTQLVALDGAELSFPSHYRRFTVATFALLHPGSQRALRGWVYFF 512
Walrus       QWLLLSDGCPFDGDSYRTRLVAVDEAELSFPSHYQRFTVATFALLDPGSQRPLRGWVYFF 512
             ** :**:*******.***::** * * ******::****:.****.* *:* * * ****

Tiger        CHSSACSPSGLETCSATCSSRPARQRRSYTPHSEATRPQNLVSSPGPVDFEDSSGQEPPL 581
Cat          CHSSACSPSGLETCSTTCSSRPARQRRSYNPHGEATRPQNLVSSPGPVDFEDSSGQEPPL 580
Cheetah      CHSSACCPSGLETCSTTCSSRPARQRRSYTPRGMATRPQNLVSSPGPVDFEDSSGQEPPL 576
Panda        CSASACTPSGLETCSTTCSSGPARRRRAYAPHSNDAERQNLVSSPGPVGFEGSYRQKPPP 585
Polar bear   CSASACSPSGLETCSTTCSSGPARQRRAYTPHSKAAERQNLVSSPGPVGFEGFYRQEPLP 587
Ferret       CSASACSPSGLETCPTMCSSGPSRQRRSSAARSTAAGPQNLVSSPGPVGFEDSYRQEPAL 572
Walrus       CSVSACSPSELETCRTVCSSGPARQRRSYAPHSKAARPQNLVSSPGPVGFEDSSRQEPPP 572
             *  *** ** **** : *** *:*:**:   :.  :  ********** **    *:*

Tiger        GPTGSPRNANQRPLLWVVLLLVAVALVLGVGVFAGLSQAKPRSSRRVTEGDWAQ------ 635
Cat          GPTGSPRNANQRPLLWVVLLLVAVALVLGVGVFEGLSQAKAQKLQEGDRGRLGSIKHRVQ 640
Cheetah      GPTGSPRNANQRPLLWVVLLLVAVALVLGVGVLAGLSEAXAQKLQEGDRGRLGSIKHRVQ 636
Panda        GTTGSPRNTDQRPLLWVVLLLVAVALVLGVGVFAGLTQAXAQKLQEDNRG---------- 635
Polar bear   RTTGSPRSTDQRPLLWVVLLLVAVALVLGVGVFAGLTQAKXQKLQEDNRGXRGSINRRLR 647
Ferret       GPTGSPRNVNQRPLLWVVLLLAAVALVLGVGVFVGLHQAKHGSSRKATEGEGAQ------ 626
Walrus       GPTGFPRNANPGPLLWVVLLLVAVALVLGVGVFVRLSRAQHRNSRKAMAGEGAQ------ 626
               ** **..:  *********.**********:  * .*   . :.   *

Tiger        ------------
Cat          PAQRVWKAIRGD 652
Cheetah      PAQRVWKAIRGD 648
Panda        ------------
Polar bear   PTKRVWKAVCGD 659
Ferret       ------------
Walrus       ------------
```

Figure 3

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                                                                      EXON 1
                      ----------------------------------------------------------------------------------------------------
Walrus                ATGGCAGGAGCCTCGGCCGGGGTCTGGGATTGCCACGTGGCCCTGCTGCTGGTGACCGCTCTGGGGCTGGGGCAGCGGCTACACCCCAAGCCTGGTCTCT 100
                       M  A  G  A  S  A  G  V  W  D  C  H  V  A  L  L  L  V  T  A  L  G  L  G  Q  R  L  H  P  K  P  G  L
Weddell seal          ACGGCAGGAGCCTCGGCCGGGGTCTGGGATTGCCACGTGGCCCTGCTGCTGATGAGCGCTCTGGGGCTGGGGCAGCAGCTACTCCCCGAGCCTTATCTCT 100
                       T  A  G  A  S  A  G  V  W  D  C  H  V  A  L  L  L  M  S  A  L  G  L  G  Q  Q  L  L  P  E  P  Y  L
Antarctic fur seal    ATGGCAGGAGCCTCGGCCGGGGTCTGGGATTGCCACGTGGCCCTGCTGCTGGTGACTGCTCTGGGGCTGGGGCAGCGGCTACACCCCAAGCCTGGTCTCT 100
                       M  A  G  A  S  A  G  V  W  D  C  H  V  A  L  L  L  V  T  A  L  G  L  G  Q  R  L  H  P  K  P  G  L

                      ---------------------------------------------------------------------------------------------
Walrus                CCAGCCTGGGGTACAGCTATGACTGTGGGGTCAAGGGCCTGCAGCTACGGGTGCTCCCCCAGTCAGGCCAGATGGTCCGCTTCAAGGTGGTAGATGAATT 200
                      S  S  L  G  Y  S  Y  D  C  G  V  K  G  L  Q  L  R  V  L  P  Q  S  G  Q  M  V  R  F  K  V  V  D  E  F
Weddell seal          CCGGCCTGGGGTACAGCTATGACTGTGGGGTCAAGAGTTTGCAGCTACGAGTGCTCCCCCGGTCAGGCCAGACAGTCCACTTCAAGGTGGTAGATGAATT 200
                      S  G  L  G  Y  S  Y  D  C  G  V  K  S  L  Q  L  R  V  L  P  R  S  G  Q  T  V  H  F  K  V  V  D  E  F
Antarctic fur seal    CCAGCCTGGGGTACAGCTATGACTGTGGGGTCAAGGGCCTGCAGCTACGGGTGCTCCCCCAGTCAGGCCAGACGGTCCGCTTCAAGGTGGTAGATGAATT 200
                      S  S  L  G  Y  S  Y  D  C  G  V  K  G  L  Q  L  R  V  L  P  Q  S  G  Q  T  V  R  F  K  V  V  D  E  F


                                                                      EXON 2
Walrus                TGGGAACCAATTTGAGGTGAACAACTGCTCTGCTTGCTACCACTGGGTCAGCACCAAGCCCCAGGCACCCGCTGTCTTCTCTGCAGGTTACAAAGGCTGC 300
                        G  N  Q  F  E  V  N  N  C  S  A  C  Y  H  W  V  S  T  K  P  Q  A  P  A  V  F  S  A  G  Y  K  G  C
Weddell seal          TGGGAACCGGTTTGAGGTAAACAACTGCTCTGCTTGTTACCACTGGGTCAGCACCAAGCCCCGGGCACCTGCTGTCTTCTCTGTGGGTTACAAAGGCTGC 300
                        G  N  R  F  E  V  N  N  C  S  A  C  Y  H  W  V  S  T  K  P  R  A  P  A  V  F  S  V  G  Y  K  G  C
Antarctic fur seal    TGGGAACCAATTTGAGGTGAACAACTGCTCTGCTTGCTACCACTGGGTCAGCACCAAGCCCTGGGCACCCGCTGTCTTCTCTGCAGGTTACAAAGGCTAC 300
                        G  N  Q  F  E  V  N  N  C  S  A  C  Y  H  W  V  S  T  K  P  W  A  P  A  V  F  S  A  G  Y  K  G  Y


                                                                      EXON 3
                                     -------------------------------------------------------------------------------------
Walrus                CACATGCTGGAGAAGGACGGGCGCTCCCACCTAACGGTGTTCATTGAAGCCGTGGGGCCCGATGGTCGAGTTGATGCAACCCGAGATGTCACTCTGATTT 400
                       H  M  L  E  K  D  G  R  S  H  L  T  V  F  I  E  A  V  G  P  D  G  R  V  D  A  T  R  D  V  T  L  I
Weddell seal          CACATGCTGGAGAAGGATGGGCGCTCCCACCTGAGGGTGCTCATCGAAGCCGTGCTGCCCGATGGTCGAGTTGATGCAACACAAGATGTCACTCTGATTT 400
                       H  M  L  E  K  D  G  R  S  H  L  R  V  L  I  E  A  V  L  P  D  G  R  V  D  A  T  Q  D  V  T  L  I
Antarctic fur seal    CACGTGCTGGAGAAGGACGGGCGCTCCCACCTGACGGTGTTCACAGAAGCCGTGCTGCCCGATGGTCGAGTTGATGCAACCCGAGATGTCGCTCTGATTT 400
                       H  V  L  E  K  D  G  R  S  H  L  T  V  F  T  E  A  V  L  P  D  G  R  V  D  A  T  R  D  V  A  L  I


                      ----------------------------------------------------------------------------------------------------
Walrus                GTCCTAAACCTGGCCACGCCTGGACTCCGGCCTCCCGTCCGGAACCACCCGTGGGCTTCTCCCTTCCCACGCCTCAGGCCCGGCCCCTCCGCCCCATCCC 500
                      C  P  K  P  G  H  A  W  T  P  A  S  R  P  E  P  P  V  G  F  S  L  P  T  P  Q  A  R  P  L  R  P  I  P
Weddell seal          GTCCTAAACCTGGCCACGCCTGGACTCCG-ACTCCCATCCGGCACCACCCATGGGCTTCTCCCTTCCCAGTCCTCAGGCCCGGCCCCTCCGCCCCATCCC 499
                      C  P  K  P  G  H  A  W  T  P   T  P  I  R  H  H  P  W  A  S  P  F  P  V  L  R  P  G  P  S  A  P  S
Antarctic fur seal    GTCCTAAACCTGGCCACGGCTGGACTCCGGCCTCCCATCCGGAACCACCCGTGGGCTTCTCCCTTCCCACGCCTCAGGCCCAGCCCCTCCGCCCCATCCC 500
                      C  P  K  P  G  H  G  W  T  P  A  S  H  P  E  P  P  V  G  F  S  L  P  T  P  Q  A  Q  P  L  R  P  I  P


                      ----------------------------------------------------------------------------------------------------
Walrus                AGAGCACGGCTTTGTCCGTGCAACCCCTGCCTTGCCGTCCCTCGAACCTGGACCCACCACCCATCCCACCCAGGCTCAACCCCAGTGGGGCACCCTGGAA 600
                        E  H  G  F  V  R  A  T  P  A  L  P  S  L  E  P  G  P  T  T  H  P  T  Q  A  Q  P  Q  W  G  T  L  E
Weddell seal          AGAGCACGGCTTTGTCTGTGCACCCCCTGCCTTGCTGTCCCTCGGACCTGGACCCACCGCCCATCCCA----GGTTAAACCCCAGTGGGGCACCCTGGAA 595
                      Q  S  T  A  L  S  V  H  P  L  P  C  C  P  S  D  L  D  P  P  P  I  P      G  *  T  P  V  G  H  P  G
Antarctic fur seal    AGAGCACAGCTTTGTCCGTGCAACCCCTGCTTTGCCGTCCCTCGGACCTGGACCCGCCACCCATCCCACCCAGGCTCAACCCCAGTGGGGCACCCTGGAA 600
                        E  H  S  F  V  R  A  T  P  A  L  P  S  L  G  P  G  P  A  T  H  P  T  Q  A  Q  P  Q  W  G  T  L  E

                      -------------------------------
Walrus                CATGGGGGGGTTGACAAGCCACCTTACCCAGGTATGCGTCTGACTCCAGGGCGGTGCCAGGTGTCCTCCAGACCCATCCCCTGTGGAGTGAGAAGTTCAG 700
                       H  G  G  V  D  K  P  P  Y  P  G  M  R  L  T  P  G  R  C  Q  V  S  S  R  P  I  P  C  G  V  R  S  S
Weddell seal          CACCGGGGGGTGGACGAGCCACCTTACCCAGGTGTGCGTCTGACTCCGGGGCGGTGCCAGGTGTCCTCCAGACCCATCCCCTGTGGAGTGAGAAGTTCAG 695
                      T  P  G  G  G  R  A  T  L  P  R  C  A  S  D  S  G  A  V  P  G  V  L  Q  T  H  P  L  W  S  E  K  F  R
Antarctic fur seal    CAC-GGGGGGTTGACGAGCCACCTTACCCAGGTGTGCGTCTGACTCCAGGGCGGTGCCAGGTGTCCTCCAGACCCATCCCCTGTGGAGTGAGAAGTTCAG 699
                       H   G  G  L  T  S  H  L  T  Q  V  C  V  *  L  Q  G  G  A  R  C  P  P  D  P  S  P  V  E  *  E  V  Q
                                                                      EXON 4
                                                                                           ---------------------------
Walrus                AAGAAGCCTGTCTGCGGGCAGGCTGCTGCTATGACAAC-AGCAGAGAGGTTCCCTGTTACTATGGCAACACAGCAACTGTCCAGTGCTTCAGAAATGGCC 799
                      E  E  A  C  L  R  A  G  C  C  Y  D  N   S  R  E  V  P  C  Y  Y  G  N  T  A  T  V  Q  C  F  R  N  G
Weddell seal          AGGAAGCCTGTCTGCAGGCGGGCTGCTGCTTTGACAACGGGCGGAGAGATTCCCTGTTACTATGGCAACACAGCAACTGTCCAGTGCTTCAGAAATGGCC 795
                        G  S  L  S  A  G  G  L  L  L  *  Q  R  A  E  R  F  P  V  T  M  A  T  Q  Q  L  S  S  A  S  E  M  A
Antarctic fur seal    AGGAAGCCTGTCTGCCGGC-GGCTGCTGCTATGACAAC-GGCAGAGAGGTTCCCTGTTACTATGGCAACACAGCAACTGTCCAGTGCTTCAGAAATGGCC 797
                       R  K  P  V  C  R   R  L  L  L  *  Q   R  Q  R  G  S  L  L  L  W  Q  H  S  N  C  P  V  L  Q  K  W  P

                                                                      EXON 5
                      ----------------------------------------------------------------------------------------------------
Walrus                ACTTTGTCCTGGTGGTGTCCCGAGAAACAGCCTTGGCACATGGGATCACACTGGCCAACATCCACATGGCCTATGCCCCCACCAGCTGCTCCCCAGCCCA 899
                      H  F  V  L  V  V  S  R  E  T  A  L  A  H  G  I  T  L  A  N  I  H  M  A  Y  A  P  T  S  C  S  P  A  Q
Weddell seal          ACTTTGTCCTGGTGGTGTCCGGAGAAACAGCCTTGGCACGTGGGATCACACTGGCCAACATCCACACGGCCTGTGCCCCCACCAGCTGCTCCCCAGCCCA 895
                       T  L  S  W  W  C  P  E  K  Q  P  W  H  V  G  S  H  W  P  T  S  T  R  P  V  P  P  P  A  A  P  Q  P
Antarctic fur seal    ACTTTGTCCTGGTGGTGTCCCGAGAAACAGCCTTGGCACATGGGATCACACTGGCCAACATCCACATGGCCTATGCCCCCACCAGCTGCTCCCCGGCCCA 897
                        L  C  P  G  G  V  P  R  N  S  L  G  T  W  D  H  T  G  Q  H  P  H  G  L  C  P  H  Q  L  L  P  G  P


                      -------------------------------------------------------------
Walrus                GAAGACCGGGTCCTTCGTGGTCTTTCGCTTCCCTTTCTCCCACTGTGGGACCACGGTCCAGGTGGCTGGCAACCAGCTCATCTATGAGAATCAGCTGGTG 999
                        K  T  G  S  F  V  V  F  R  F  P  F  S  H  C  G  T  T  V  Q  V  A  G  N  Q  L  I  Y  E  N  Q  L  V
Weddell seal          GGAGACCGGGTCCTTCGTGGTCTTTCGCTGCCCTTTCTCCCACTGTGGGACCACGGTCCAGGTGGCCGGCAACCAGCTCATCTATGAGAATCAGCTGGTG 995
                      R  R  P  G  P  S  W  S  F  A  A  L  S  P  T  V  G  P  R  S  R  W  P  A  T  S  S  S  M  R  I  S  W  C
Antarctic fur seal    GCAGACCGGGTCCTTCGTGGTCTTTCGCTTCCCTTTCTCCCACTGTGGGACCACGGTCCAGGTGGCTGGCAACCAGCTCATCTATGAGAATCAGCTGGTG 997
                       A  D  R  V  L  R  G  L  S  L  P  F  L  P  L  W  D  H  G  P  G  G  W  Q  P  A  H  L  *  E  S  A  G


                                                                      EXON 6
                                                                                 -----------------------------------------
Walrus                TCTGACATTGAGGCCCAAACGGGGCCACAGGGCTCCATCACGCGGGACGGCACCTTCCGGCTTCACGTGCGCTGCGTCTTTAACACCAGTGACTTCCTGC 1099
                       S  D  I  E  A  Q  T  G  P  Q  G  S  I  T  R  D  G  T  F  R  L  H  V  R  C  V  F  N  T  S  D  F  L
Weddell seal          TCTGACTTCGAGGCCCAAACGGGGCCACAGGGCTCCATCACGCGGGATGGCACCTTCTGGCTTCACGTGTGCTGCGTCTTCAACACTGGTGATTTCCTGC 1095
                        L  T  S  R  P  K  R  G  H  R  A  P  S  R  G  M  A  P  S  G  F  T  C  A  A  S  S  T  L  V  I  S  C
Antarctic fur seal    TCTGACATCAAGGCCCAAACGGGGCCACAGGGCTCCATCACGCGGGACGGCACCTTCCGGCTTCACGTGTGCTGCGTCTTTAACGCCAGTGACTTCCTGC 1097
                      V  *  H  Q  G  P  N  G  A  T  G  L  H  H  A  G  R  H  L  P  A  S  R  V  L  R  L  *  R  Q  *  L  P  A
```

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                                                                      EXON 7
                      ---------------------------------------------------------------------------------------
Walrus                CGCTCCAGGCGTCCATCTTCCTGCCGCCCTCGCCAGCCCCTGTGACCCAGTCCGGCCCCCTGCAGCTCCAGCTTCGGATCGCCAAGGATGAGAGTTTCCG 1199
                      P  L  Q  A  S  I  F  L  P  P  S  P  A  P  V  T  Q  S  G  P  L  Q  L  Q  L  R  I  A  K  D  E  S  F  R
Weddell seal          CGCTCCGGGCGTCCATCTTCCTGCCGCCCTTGCCAGTCCCCATGACCCAGTCCGGCCCCCTGCGGCTCCAGCTTCGGATCGCCAAGGATGAGACTTTCCG 1195
                       R  S  G  R  P  S  S  C  R  P  C  Q  S  P  *  P  S  P  A  P  C  G  S  S  F  G  S  P  R  M  R  L  S
Antarctic fur seal    CGCTCCGGGCGTCCATCTTCCTGCCACCCTCGCCAGCCCCTGTGACCCAGTCCGGCCCCCTGCAGCTCCAGCTTCGGATCGCCAAGGATGAGAGTTTCCG 1197
                        A  P  G  V  H  L  P  A  T  L  A  S  P  C  D  P  V  R  P  P  A  A  P  A  S  D  R  Q  G  *  E  F  P

                                                                      EXON 8
Walrus                CTCCTACTACGAGGAGGGGGACTACCCCCTCGTGAGGCTGCTGCGCCAGCCTGTCCCGGTGGAGGTCCGGCTCCTGGAGAGGACAGACCCCAGTCTGGTC 1299
                        S  Y  Y  E  E  G  D  Y  P  L  V  R  L  L  R  Q  P  V  P  V  E  V  R  L  L  E  R  T  D  P  S  L  V
Weddell seal          CTCCTACTACAGGGAGGGGGACTACCCCCTCGTGAGGCTGCTGCGCGAGCCTGTCCTGGTGGAGGTCTGGCTCCTGCAGAGGACAGACCCCAGTCTGGCC 1295
                      A  P  T  T  G  R  G  T  T  P  S  *  G  C  C  A  S  L  S  W  W  R  S  G  S  C  R  G  Q  T  P  V  W  P
Antarctic fur seal    CTCCCACTACGAGGAGGGGGACTACCCCCTCGTGAGGCTGCTGCGCCAGCCTGTCCCAGTGGAGGTCTGGCTCCTGCAGAGGACAGACCCCAGTCTGGTC 1297
                       L  P  L  R  G  G  G  L  P  P  R  E  A  A  A  P  A  C  P  S  G  G  L  A  P  A  E  D  R  P  Q  S  G

                                                                                             -----------------------
Walrus                CTGCTGCTGCACCAGTGCTGGGCGACTCCCGGTGCCAACCCCTTCCAGCAGCCTCAGTGGCTCCTCCTGTCTGACGGGTGTCCTTTTGATGGTGACAGCT 1399
                       L  L  L  H  Q  C  W  A  T  P  G  A  N  P  F  Q  Q  P  Q  W  L  L  L  S  D  G  C  P  F  D  G  D  S
Weddell seal          CTGCTGCTGCAC-AGAGCCAGGCCGCCCCCGGTGCCAGCCCCCTCCAGCAGCCTCAGCGGCCCCTCCCGTCTGACGGGTGTCCTTTCGACAGTGACAGCT 1394
                        C  C  C  T   E  P  G  R  P  R  C  Q  P  P  P  A  A  S  A  A  P  P  V  *  R  V  S  F  R  Q  *  Q  L
Antarctic fur seal    CTGCTGCTGCACCAGTGCTGGGCGACTCCCGGTGCCAACCCCTTCCAGCAGCCTCAGTGGCTCCTCCTGTCTGACGGGTGTCCTTTTGACTGTGACAGCT 1397
                      P  A  A  A  P  V  L  G  D  S  R  C  Q  P  L  P  A  A  S  V  A  P  P  V  *  R  V  S  F  *  L  *  Q  L


                                                                      EXON 9
                      ----------------------------------------------------------------------------------------------------
Walrus                ACAGGACCCGACTGGTAGCCGTGGACGAGGCAGAACTGTCCTTCCCATCCCACTACCAGCGCTTCACCGTTGCCACCTTCGCCCTCCTGGACCCTGGCTC 1499
                      Y  R  T  R  L  V  A  V  D  E  A  E  L  S  F  P  S  H  Y  Q  R  F  T  V  A  T  F  A  L  L  D  P  G  S
Weddell seal          ACAGGTCCCGACTGGTAGCCTTGGATGGGGCAGAGCTGTCCTTCCCATCCCACTACCAGCGCTTCATCGTTGCCACCTTCGCCCTCCTGGACCCTGGCTC 1494
                        Q  V  P  T  G  S  L  G  W  G  R  A  V  L  P  I  P  L  P  A  L  H  R  C  H  L  R  P  P  G  P  W  L
Antarctic fur seal    ACAGGACCCAACTGGTAGCCTTGGACGAGGCAGAACTGTCCTTCCCATCCCGCTACCAGCACTGCACCGTTGCCACCTTTGCCCTCCTGGACCCTGGCTC 1497
                        Q  D  P  T  G  S  L  G  R  G  R  T  V  L  P  I  P  L  P  A  L  H  R  C  H  L  C  P  P  G  P  W  L

                                                                      EXON 10
                      ----------------------
Walrus                CCAGAGGCCCCTCCGGGGATGGGTCTACTTCTTCTGCAGCGTCTCTGCCTGCTCCCCTTCGGAGCTGGAGACGTGCCGCACTGTTTGCAGCTCTGGGCCT 1599
                        Q  R  P  L  R  G  W  V  Y  F  F  C  S  V  S  A  C  S  P  S  E  L  E  T  C  R  T  V  C  S  S  G  P
Weddell seal          CCAGAGGCCCCTCCGGGGATGGGTTTACTTTTTCTGCAGCGTCTCTGCCTGCTCCCCTTCGGGGCTGGGGACGTGCCCCACTGTGTGCAGCTCTGGGCCT 1594
                       P  E  A  P  P  G  M  G  L  L  F  L  Q  R  L  C  L  L  P  F  G  A  G  D  V  P  H  C  V  Q  L  W  A
Antarctic fur seal    CCAGAGGCCCCTCCGGGGATGGGTTTACTTCTTCTGCAGCGTCTCTGCCTGCTCCCCTTCGGAGCTGGAGACGTGCCGCACTGTTTGCAGCTCTGGGCTT 1597
                       P  E  A  P  P  G  M  G  L  L  L  L  Q  R  L  C  L  L  P  F  G  A  G  D  V  P  H  C  L  Q  L  W  A

                                                                      EXON 11
                          ------------------------------------------------------------------------------------------------
Walrus                GCAAGACAGCGACGATCCTACGCTCCCCACAGCAAAGCTGCCAGGCCCCAGAACCTCGTGAGCTCTCCAGGGCCAGTGGGCTTTGAGGATTCTTCCAGGC 1699
                       A  R  Q  R  R  S  Y  A  P  H  S  K  A  A  R  P  Q  N  L  V  S  S  P  G  P  V  G  F  E  D  S  S  R
Weddell seal          GCGAGACAGCGACGATCCTACGCTCCCCACAGCAAAGCTGCCAGGCCCCAGAACCTCATGAGCTCTCCAGGGCCAGTGGGCTTTGAGGATTCTTCCAGGC 1694
                      C  E  T  A  T  I  L  R  S  P  Q  Q  S  C  Q  A  P  E  P  H  E  L  S  R  A  S  G  L  *  G  F  F  Q  A
Antarctic fur seal    GCAAGACAGCGACGATCCTACGCTCCCCACAGAAAAGCTGCCAGGCCCCAGAACCTCGTGAGCTCTCCAGGGCCAGTGGGCTTTGAGGATTCTTCCAGGC 1697
                      C  K  T  A  T  I  L  R  S  P  Q  K  S  C  Q  A  P  E  P  R  E  L  S  R  A  S  G  L  *  G  F  F  Q  A

                                                                      EXON 12
                      ------------------------
Walrus                AGGAGCCTCCGCCGGGGCCCACAGGCTTCCCCAGGAACGCCAACCCGGGGCCTCTCCTCTGGGTGGTCCTTCTGCTGGTGGCTGTTGCCCTGGTCCTGGG 1799
                      Q  E  P  P  P  G  P  T  G  F  P  R  N  A  N  P  G  P  L  L  W  V  V  L  L  L  V  A  V  A  L  V  L  G
Weddell seal          AGGAGCCTCTGCCGGGGCCCACAGGCTCCCCCGGGAACGCCAACCCGGGGCCTCTCCTCTGGGTGGTCCT---GCTGGTGGCTGTTGCCCTGGTCCTGGG 1791
                        G  A  S  A  G  A  H  R  L  P  R  E  R  Q  P  G  A  S  P  L  G  G  P     A  G  G  C  C  P  G  P  G
Antarctic fur seal    AGGAGCCTCCGCCGGGGCCCACAGGCTTCCCCAGGAATGCCAACCCGGGGCCTCTCCTCTGGGTGGCCCTTCTGCTGGTGGCTGTTGCCCTGGTCTTGGG 1797
                        G  A  S  A  G  A  H  R  L  P  Q  E  C  Q  P  G  A  S  P  L  G  G  P  S  A  G  G  C  C  P  G  L  G


Walrus                AGTCGGTGTTTTCGTGCGCCTGAGCCGAGCCCAGCACAGGAACTCCAG-GAAGGCAATGGCGGGTGAAGGGGCTCAATAA 1878
                        V  G  V  F  V  R  L  S  R  A  Q  H  R  N  S  R   K  A  M  A  G  E  G  A  Q  *
Weddell seal          GGTCAGTGTTTTCATGCGCCTGAGCCGAGCCCAGCGCAGGAGTTCCAGTGAAGGCAACGGAGGGTGAAGGGGCTCAATAA 1871
                       G  Q  C  F  H  A  P  E  P  S  P  A  Q  E  F  Q  *  R  Q  R  R  V  K  G  L  N
Antarctic fur seal    GGTCGGTGTTTTCGTGCGCCTGAGCCGAGCCCAGCACAGGAGCTCCAG-GAAGGCAATGGAGGGTGAAGGGGCTCAATAA 1876
                       G  R  C  F  R  A  P  E  P  S  P  A  Q  E  L  Q   E  G  N  G  G  *  R  G  S  I
```

Figure 4

```
   1 atggcagggatctcggccaggctccgggacggttgcgtggcgctgctgctggtggctgct
   1  M  A  G  I  S  A  R  L  R  D  G  C  V  A  L  L  L  V  A  A
  61 ctggggctgacgcagcggccacacaccgaacctggtccctcaggcctgtggcacggctat
  21  L  G  L  T  Q  R  P  H  T  E  P  G  P  S  G  L  W  H  G  Y
 121 gactgtggggtcaagggcatgcagctatgggccttcccggggccaggccagacaatccgc
  41  D  C  G  V  K  G  M  Q  L  W  A  F  P  G  P  G  Q  T  I  R
 181 ttcaaggtggtagatgaatttgggaaccaatttgaggtaaacaactgttctgcctgctac
  61  F  K  V  V  D  E  F  G  N  Q  F  E  V  N  N  C  S  A  C  Y
 241 cactgggtcaccaccaagcccccgggacacgcggtcttctctgctggttacaaaggctgc
  81  H  W  V  T  T  K  P  P  G  H  A  V  F  S  A  G  Y  K  G  C
 301 cacgtgctggagaaggacggcgctcccacctgaaggtgatcatcgaagccgtgctgccc
 101  H  V  L  E  K  D  G  R  S  H  L  K  V  I  I  E  A  V  L  P
 361 aacggtcaagttgaggcaacaggagatgtcactctgatttgtcctaaacctgcccacacc
 121  N  G  Q  V  E  A  T  G  D  V  T  L  I  C  P  K  P  A  H  T
 421 tggactccggacccacacctggcaccacgcacaggcttctcccgccccaccccccaggcc
 141  W  T  P  D  P  H  L  A  P  R  T  G  F  S  R  P  T  P  Q  A
 481 tggtccctccgccccaacccagagcacagcttcgtccatgcgacccctgccttgccgtcc
 161  W  S  L  R  P  N  P  E  H  S  F  V  H  A  T  P  A  L  P  S
 541 ctcggacctggacccacctcccatgccacccaggccccaccccagggggcaccctgaga
 181  L  G  P  G  P  T  S  H  A  T  Q  A  P  P  Q  G  G  T  L  R
 601 ccctgggggttgacgagccaccatactcaggtgcacctctgactccagagctgtgccag
 201  P  W  G  V  D  E  P  P  Y  S  G  A  P  L  T  P  E  L  C  Q
 661 gtgccctcaagggccatctcctgtggagtgggaagaagctcgaaggaagcctgccagcag
 221  V  P  S  R  A  I  S  C  G  V  G  R  S  S  K  E  A  C  Q  Q
 721 gctggctgctgctatgacaacagcagagcgattccctgttactatggcaacacagcaact
 241  A  G  C  C  Y  D  N  S  R  A  I  P  C  Y  Y  G  N  T  A  T
 781 gtccagtgcttcagaaatggccactttgtcctggtggtgtcccaagaaactgccttggcg
 261  V  Q  C  F  R  N  G  H  F  V  L  V  V  S  Q  E  T  A  L  A
 841 cacgggatcacgctggccaacctccacatggcctatgcccccacYggctgctcccccacc
 281  H  G  I  T  L  A  N  L  H  M  A  Y  A  P  T  G  C  S  P  T
 901 caggagaccgggtccttcgtggtcttccgcttcccctctcccactgtgggaccacagcc
 301  Q  E  T  G  S  F  V  V  F  R  F  P  L  S  H  C  G  T  T  A
 961 caggtggctggcaaccagctcgtctatgagaatcagctggtgtctgacatcgaggctcgg
 321  Q  V  A  G  N  Q  L  V  Y  E  N  Q  L  V  S  D  I  E  A  R
1021 acggggccacagggctccatcacaagggacggcaccttccggcttcacatgcgctgcatc
 341  T  G  P  Q  G  S  I  T  R  D  G  T  F  R  L  H  M  R  C  I
1081 ttcaacgccagtgacttcctgccgctccaggcatccatcttcccgccaccctctccagcc
 361  F  N  A  S  D  F  L  P  L  Q  A  S  I  F  P  P  P  S  P  A
1141 cctgtgacccagtccgggcccctgcatctccagcttcggatcgccaaagatgagactttc
 381  P  V  T  Q  S  G  P  L  H  L  Q  L  R  I  A  K  D  E  T  F
1201 cgctccttctacgaggaaggggactacccccctcgtgaggctgctgcgtgagcctgtccca
 401  R  S  F  Y  E  E  G  D  Y  P  L  V  R  L  L  R  E  P  V  P
1261 gtggaggtccggctcctgcacaggacagaccccggYctggtcctgctgctgcaccagtgc
 421  V  E  V  R  L  L  H  R  T  D  P  G  L  V  L  L  L  H  Q  C
1321 tgggccactcccggggccagccctttccagcagcctcagtggcccattctgtctgaaggg
 441  W  A  T  P  G  A  S  P  F  Q  Q  P  Q  W  P  I  L  S  E  G
1381 tgtccttttgatggcgacagctacaggacccaactggtagccttggacggggcagagctt
 461  C  P  F  D  G  D  S  Y  R  T  Q  L  V  A  L  D  G  A  E  L
1441 tccttcccatcccactaccggcgcttcaccgtggccaccttcgccctcctgcaccctggc
 481  S  F  P  S  H  Y  R  R  F  T  V  A  T  F  A  L  L  H  P  G
1501 tcccagagggccctcaggggatgggtttacttcttctgcagtgcctctgcctgttcccct
 501  S  Q  R  A  L  R  G  W  V  Y  F  F  C  S  A  S  A  C  S  P
1561 tcggggctggagacctgccccactatgtgcagctctgggccctcgagacagcgacgatcc
 521  S  G  L  E  T  C  P  T  M  C  S  S  G  P  S  R  Q  R  R  S
1621 tctgctgcccgcagcactgctgctgggccccagaaccttgtgagctctccagggcccgtg
 541  S  A  A  R  S  T  A  A  G  P  Q  N  L  V  S  S  P  G  P  V
1681 ggctttgaggattcttacaggcaggagcctgcgctggggcccacaggctcccccaggaac
```

```
 561  G  F  E  D  S  Y  R  Q  E  P  A  L  G  P  T  G  S  P  R  N
1741 gtcaaccagaggcctctcctctgggtggtccttctgctggcggctgttgccctggtccta
 581  V  N  Q  R  P  L  L  W  V  V  L  L  L  A  A  V  A  L  V  L
1801 ggggtcggtgttttcgtgggcctgcaccaagccaagcacggaagctccaggaaggccaca
 601  G  V  G  V  F  V  G  L  H  Q  A  K  H  G  S  S  R  K  A  T
1861 gagggcgaaggggctcaataa
 621  E  G  E  G  A  Q  -
```

Figure 5

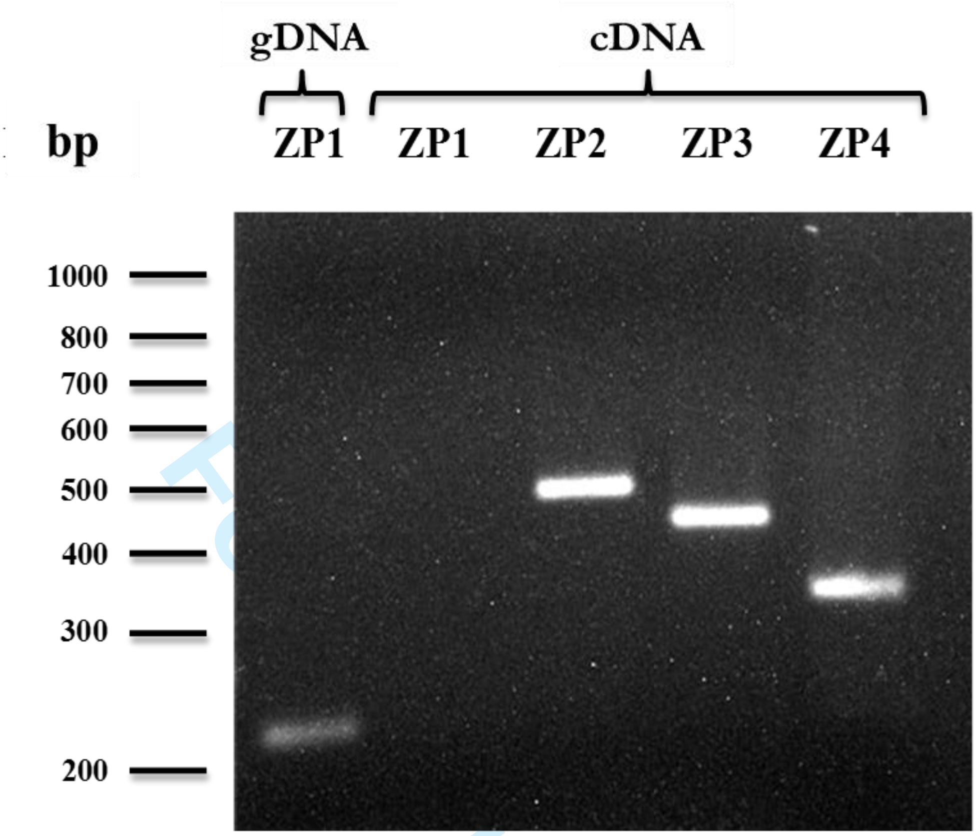
gDNA
cDNA
bp
ZP1
ZP1
ZP2
ZP3
ZP4
1000
800
700
600
500
400
300
200

Figure 6

```
   1 atgcggcagctgcagatcatcttgctctgttttcccttgtctcttgcgttgaggggccac
   1  M  R  Q  L  Q  I  I  L  L  C  F  P  L  S  L  A  L  R  G  H
  61 cctgagcctgaggcaccagattatctgggtgagctccactgtgggctccggagtcttcgg
  21  P  E  P  E  A  P  D  Y  L  G  E  L  H  C  G  L  R  S  L  R
 121 ttcaccgtaaacctgagccaggggacagcgactcctacgctaatagcttgggatgaccac
  41  F  T  V  N  L  S  Q  G  T  A  T  P  T  L  I  A  W  D  D  H
 181 gggctgccacgcaggctgcagaatgactctggctgtggtacctgggtgacggagggccca
  61  G  L  P  R  R  L  Q  N  D  S  G  C  G  T  W  V  T  E  G  P
 241 ggaagctccatggtgttagaagcctcttatgatggctgctatgtcaccgagtgggtgagg
  81  G  S  S  M  V  L  E  A  S  Y  D  G  C  Y  V  T  E  W  V  R
 301 acgactcgatcaccagaaatgccaaggccccgtgcgtcaccatcaggggtgtctccccag
 101  T  T  R  S  P  E  M  P  R  P  R  A  S  P  S  G  V  S  P  Q
 361 gacccccactatatcatgatggttggagttgaaggagcagatgtggctggatgcaacatg
 121  D  P  H  Y  I  M  M  V  G  V  E  G  A  D  V  A  G  C  N  M
 421 gttaccaagacacagctgctcaggtgtcctatggatccccccagacccaactttgttatct
 141  V  T  K  T  Q  L  L  R  C  P  M  D  P  P  D  P  T  L  L  S
 481 agcttgagttactctcctgatcaaaacagagccctagatgtcccaaatgctgatctgtgt
 161  S  L  S  Y  S  P  D  Q  N  R  A  L  D  V  P  N  A  D  L  C
 541 gactttgtcccagtgtgggacaggctgccatgtgttccttcacccatcactgaaggagac
 181  D  F  V  P  V  W  D  R  L  P  C  V  P  S  P  I  T  E  G  D
 601 tgcaagaagattggttgctgctacaattcggaggtgaatttctgttattatggaaacaca
 201  C  K  K  I  G  C  C  Y  N  S  E  V  N  F  C  Y  Y  G  N  T
 661 gtgacctcacactgtacccaagatggctacttctacatcactgtgtctcgggatgtgacc
 221  V  T  S  H  C  T  Q  D  G  Y  F  Y  I  T  V  S  R  D  V  T
 721 tcgcccccacttctcttgaattctgtgcgcttggccttcgggaatgatgtggaatgtacc
 241  S  P  P  L  L  L  N  S  V  R  L  A  F  G  N  D  V  E  C  T
 781 cctgcgatggcaacacacactttttgccctattctggtttccatttaactcctgtggtacc
 261  P  A  M  A  T  H  T  F  A  L  F  W  F  P  F  N  S  C  G  T
 841 acaagacggatcactggagaccaggcagtatatgaaaatgagctggttgcagctagagat
 281  T  R  R  I  T  G  D  Q  A  V  Y  E  N  E  L  V  A  A  R  D
 901 gttagaacttggagccatggttctatcacccgtgacagtattttcaggctccgagttagc
 301  V  R  T  W  S  H  G  S  I  T  R  D  S  I  F  R  L  R  V  S
 961 tgcagctactctataagtagcaatgccttcccagttaatgtccacgtgtttacatttcca
 321  C  S  Y  S  I  S  S  N  A  F  P  V  N  V  H  V  F  T  F  P
1021 ccaccgcattctgagacccagcctggacccctcactctggaactcaagattgccaaggat
 341  P  P  H  S  E  T  Q  P  G  P  L  T  L  E  L  K  I  A  K  D
1081 aagcactatggttccttctacactgttggtgactacccagtggtgaagctacttcgggat
 361  K  H  Y  G  S  F  Y  T  V  G  D  Y  P  V  V  K  L  L  R  D
1141 cccatttatgtggaggtctctatccgccacagaacagacccccacctggggctgctcctc
 381  P  I  Y  V  E  V  S  I  R  H  R  T  D  P  H  L  G  L  L  L
1201 cattactgttgggccacacccagcagaaacccacagcatcagccccagtggctcatgcta
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1261 gtgaaagggtgcccctacactggagacaactatcagacgcagctgattcctgtccagaaa
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1321 gtcctggatcctccatttccatcttactaccagcgcttcagcattttttaccttcagcttt
 441  V  L  D  P  P  F  P  S  Y  Y  Q  R  F  S  I  F  T  F  S  F
1381 atagactcggtgacaaagtgggcactcaggggaccggtgtatctgcactgtagtgcatcc
 461  I  D  S  V  T  K  W  A  L  R  G  P  V  Y  L  H  C  S  A  S
1441 gtctgccagcctgctggaacaccgtcctgtatgataacctgtcctgttgccaggcaaaga
 481  V  C  Q  P  A  G  T  P  S  C  M  I  T  C  P  V  A  R  Q  R
1501 agaaactctaacatccattttcacaaccatactgctagcatttctagcaagggtcccatg
 501  R  N  S  N  I  H  F  H  N  H  T  A  S  I  S  S  K  G  P  M
1561 attctactccaagccactaaagactcaggaaagctccataaatactcaagttttcctgta
 521  I  L  L  Q  A  T  K  D  S  G  K  L  H  K  Y  S  S  F  P  V
1621 gactctcaaactctgtggatggcaggcctttctgggaccttaatcgttggagccttgtta
 541 D  S  Q  T  L  W  M  A  G  L  S  G  T  L  I  V  G  A  L  L
1681 gtgtcctacttagctatcaggaaatag
 561  V  S  Y  L  A  I  R  K  -
```

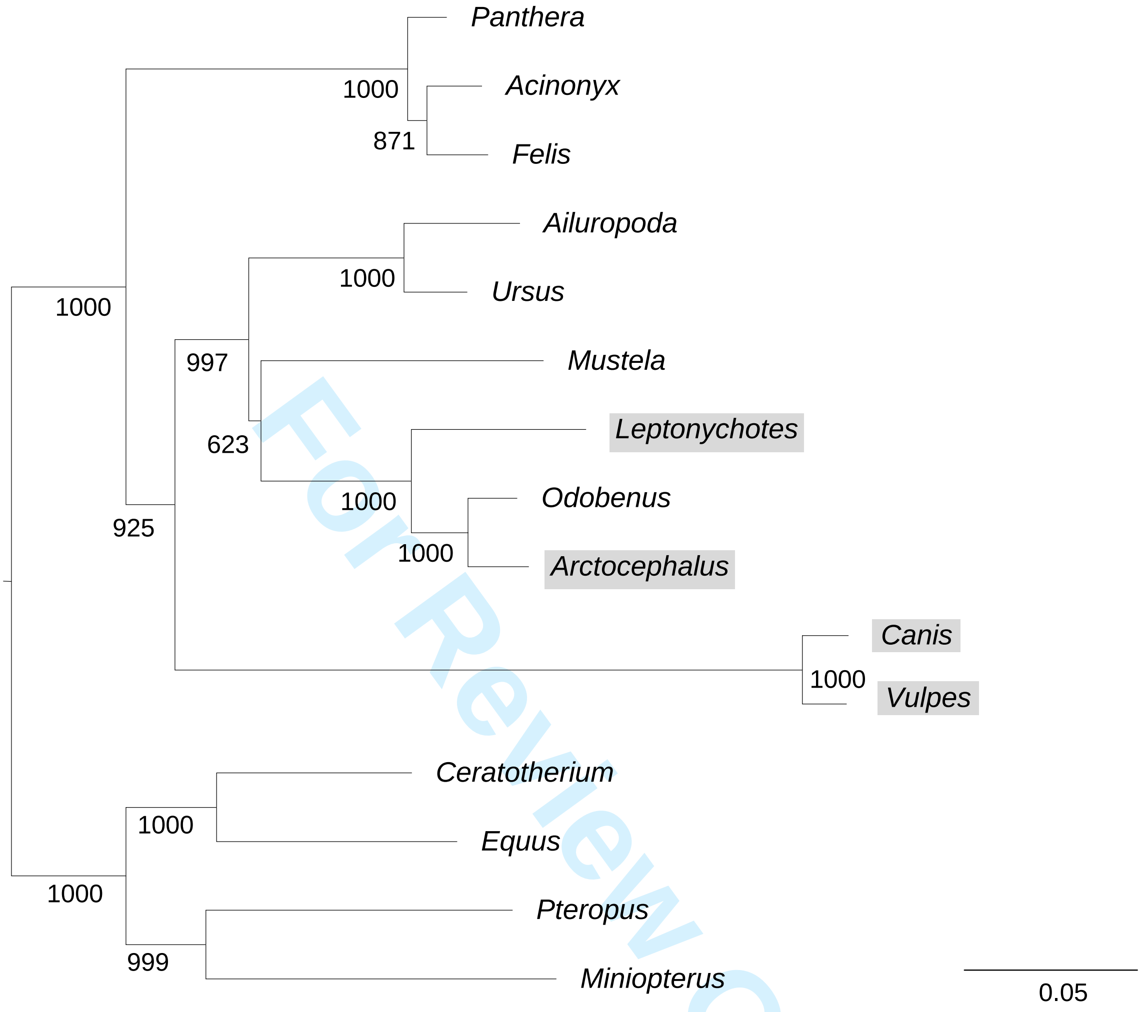
Figure 7

```
                                                                ↓
Dog           ---------QVSISPRPPPAPVSPSGPCGSSSNHQGYGAPRPAEETFCSY*EERDYPNIR
Fox           FNASSLLLLQVSIFPQPPPAPVSPSGPCGSSSNHRGYGAPRATDETFCSY*EERDYPNIR
                       **** *:******************:****** ::****************

Dog           LPCKPVPVGVRLLRAQTPVWSCCCTSAGPLPVPAPSSSLSGPSYQTDEWQGMFLLPQGVT
Fox           LPCKPVPVGVRLLRAQTPVWSCCCTSAGPLLVPAPSSSLSGPSYQTDEWQGMFLLPQGVT
              ****************************** *****************************

Dog           PPTSPIPLLPTWPLSFPGVLLTG-------------------------------------
Fox           PPTSPIPLLPTWPHSFPGVLLTGTATGPKWYPWTEVSFSSHCQCFTVTTFALPDPGSQRT
             ************* *********
```

Figure 8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Felis
Ailuropoda
1000
Ursus
1000
Mustela
997
Leptonychotes
623
Odobenus
925
1000
Arctocephalus
1000
Canis
1000
Vulpes
Ceratotherium
1000
Equus
1000
Pteropus
999
Miniopterus
0.05

Table 1. Accession numbers for the different carnivore ZP sequences.

Species	**ZP1**	**ZP2**	**ZP3**	**ZP4**
Cat	HQ702466 (Stetson et al. 2015)	U05776, D45067, NM_001009875 (Harris et al. 1994; Jewgenow and Fickel, 1999; Okazaki et al. 2007; Eade et al. 2009)	U05778, D45068, NM_001009330 (Harris et al. 1994, 1995; Okazaki and Sugimoto, 1995; Jewgenow and Fickel, 1999; Eade et al. 2009)	U05777, NM_001009260 (Harris et al. 1994; Jewgenow and Fickel, 1999; Eade et al. 2009)
Dog	**pseudogene** (Goudet et al. 2008)	U05779, NM_001003304, D45069 (Harris et al. 1994, Okazaki et al. 1995)	U05780, NM_001003224, D45070 (Harris et al. 1994, Okazaki and Sugimoto, 1995)	AY573930 (partial) (Blackmore et al. 2004; McLaughlin et al. 2004)
Ferret	KX583606*		AY702973 (Jackson and Beaton, 2004)	
Fox		AY598031 (Beaton and Bradley, 2004)	AY598032 (Reubel et al. 2005)	KF956365*
Stoat		AY779765 (partial) (Jackson and Beaton, 2004)	AY648050 (Jackson and Beaton, 2004)	AY779766 (Jackson and Beaton, 2004)
*** This study**				

Table 2. Accession numbers for the different ZP1 sequences included in the phylogenetic analysis.

Family	**Common name**	**Scientific name**	**Accession number**
Canidae	Dog	*Canis lupus*	Blast hit chromosom 18 (ENSEMBL 84)
Canidae	Fox	*Vulpes vulpes*	This study
Felidae	Cat	*Felis catus*	HQ702466
Felidae	Cheetah	*Acinonyx jubatus*	XM_015075436
Felidae	Tiger	*Panthera tigris*	XM_007092010
Mustelidae	Ferret	*Mustela putorius furo*	XM_004770464
Odobenidae	Walrus	*Odobenus rosmarus*	XM_012565789
Phocidae	Weddell seal	*Leptonychotes weddellii*	XM_006743365
Otariidae	Antarctic fur seal	*Arctocephalus gazella*	SRP064853 and Dryad: doi:10.5061/dryad.8kn8c
Ursidae	Panda	*Ailuropoda melanoleuca*	XM_002928655
Ursidae	Polar bear	*Ursus maritimus*	XM_008707545
OUTGROUPS			
Equidae	Horse	*Equus caballus*	XM_001493722
Pteropodinae	Black flying fox	*Pteropus alecto*	XM_015588713
Rhinoceratidae	White rhinoceros	*Cerathotherium simum simum*	XM_004437785
Vespertilionidae	Natal long-fingered bat	*Miniopterus natalensis*	XM_016200361