Gamete interaction protein evolution evinces Pantherines hybridizing history

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Abstract

The jaguar (*Panthera onca*) is the most relevant predator in South America and an endangered species in Argentina. Therefore genomic, evolutionary and functional studies of this species are strategic to gain biological insights and support conservation efforts. With the advent of sequencing advances the study of relevant character species evolution has to be revisited. Recent comparative genomic analysis revealed a long history of hybridization among extant big cats, which indicates an absence of inter-specific isolation barriers. We aimed to uncover the molecular basis of this inter-species crossing and the evolution of the functional capacities of pantherine gamete interaction Zona Pellucida proteins (ZP) and JUNO. Hi-C long-range sequencing and chromosome-resolved jaguar genome assembly was used to resolve ZP genes loci and reconstruct the evolutionary history of these proteins. Exploring sequence variability in more that 30 Carnivora species, Feliforme and Caniforme sister groups show significant differences in average sequence identity for all studied proteins except ZP4, always showing Caniformes as a more diverse group than Feliformes. Molecular evolution of ZP subunits involved in sperm-recognition, ZP3 and ZP2, showed similar phylogenetic histories, displaying signatures of positive selection among Carnivores and Caniformes, but not among Pantherines or Feliformes. On the other hand, structural cross-linking ZP subunits, ZP1 and ZP4, displayed a different evolutionary history. The fusion protein IZUMO1R was found to be highly conserved in all studied phylogenetic groups. These results strengthen the idea that ZPs are proteins more related to specific-specificity isolation than IZUMO1R, which is specialized in fusion events. Altogether, our findings indicate that sperm-oocyte interaction and fusion proteins lack the degree of diversification necessary to establish a prezygotic reproductive isolation barrier between pantherines, which would explain observed reticulated patterns of evolution in this clade.

1. Introduction

The zona pellucida is the extracellular matrix synthesized in the ovaries of mammals during follicular development. It is found surrounding the oocyte and early embryo plasma membrane and constitutes a three-dimensional structure composed of ZP glycoprotein filaments. Oocyte formation, fertilization and early embryo development are biological processes involving ZP proteins (Yanagimachi, 1994; Dean, 2007; Wassarman & Litscher, 2009; Gupta and Bhandari, 2011; Gupta et al., 2012; Tanihara et al., 2013; Shu et al., 2015; Moros-Nicolás et al., 2018a; Moros-Nicolás et al., 2021).

The zona pellucida of most, but not all, eutherians is composed of four ZP protein subunits: ZP1-4.

ZP3 was the first glycoprotein of the zona pellucida to be considered indispensable for fertilization in mammals when two groups independently discovered that mouse oocytes with a disrupted *Zp3* gene lack zona pellucida and are infertile (Liu et al., 1996; Rankin et al., 1996). Therefore, ZP3 was, for some time, regarded as the key and main sperm-binding protein.

Further research revealed that ZP2 is also a protein essential for mouse fertility as it is required for sperm binding (Rankin et al., 2001). Capacitated sperm attach to an N-terminal domain of ZP2 in the extracellular zona pellucida surrounding eggs (Baibakov et al., 2012; Avella et al., 2014). After fertilization, ovastacin, an oocyte-specific metalloendoproteases, is exocytosed from egg cortical granules and cleaves extracellular ZP2 (Burkart et al., 2012). The cleavage site for this event is located in the ZP-N2 domain of ZP2. Postfertilization proteolytic cleavage at the N terminus of ZP2 protein prevents sperm binding to the zona pellucida and thus provides a block to polyspermy (Baibakov et al., 2012).

ZP1 is a ZP protein not directly related to gamete interaction, but its function is no less relevant for the formation of the zona pellucida. ZP1 is involved in ZP protein filaments cross-linking (Monné & Jovine, 2011; Nishimura et al., 2019; Stsiapanava et al., 2020), thus giving and maintaining the structure of such extracellular matrix.

ZP4 was the last of the eutherian ZP proteins to be identified (Lefièvre et al., 2004), as it is not present in the house mouse (*Mus musculus*), the traditional mammalian model in which most studies of the zona pellucida had been performed. ZP4 is considered to be a paralog of ZP1 and the origin of these two modern genes can be traced back to a duplication of a common ancestral gene in the early evolution of amniotes (Goudet et al., 2008). Similar to ZP1, ZP4 plays a structural and mechanical role that is not essential during fertilization *in vivo* but fundamental for the protection of the developing embryo (Lamas-Toranzo et al., 2019).

The study of ZP proteins family evolutionary history has uncovered many events of gene gains and losses. The duplication that gave rise to *Zp1* and *Zp4* occurred during the early evolution of the amniotes (Hughes and Barratt, 1999; Bausek et al., 2000; Goudet et al., 2008) and multiple events of pseudogenization resulted in modern placental mammals whose zona pellucida structure is only composed of three subunits instead of four. *Zp1* was lost in some species of the Caniformia suborder, such as dog (*Canis familiaris*), dingo (*Canis lupus dingo*) and fox (*Vulpes vulpes*) (Goudet et al.,

2008; Moros-Nicolás et al., 2018a). *Zp1* gene was also lost among some Pinnipeds (Moros-Nicolás et al., 2018a) and at least one more pseudogenization of *Zp1* took place in the evolution of Cetartiodactyla as many even-toed ungulates and cetaceans lack a functional version of this gene (Goudet et al., 2008; Stetson et al., 2012). Other events of *Zp1* gene pseudogenization are reported in small primates lineages, such as marmosets and tarsiers (Stetson et al., 2012). On the other hand, pseudogenization of *Zp4* gene was described in the house mouse (*Mus musculus*) (Lefièvre et al., 2004) and other members of the *Mus* genus (Izquierdo-Rico et al., 2021). However, ZP3 and ZP2 proteins have not been reported as lacking in any mammalian species. This indicates that their function is essential and they both have been reported as necessary for gamete interaction and fertilization (Liu et al., 1996; Rankin et al., 1996, 2001).

The structure of ZP proteins is characterized by different domains crucial to the secretion and assembly of ZP subunits. These are an N-terminal secretory signal peptide (SP) that identifies secreted proteins; the ZP module, a conserved sequence of ~260 amino acids composed of a ZP-N and a ZP-C domain, and include 8 or 10 invariant cysteine residues that form four, or alternatively five, disulfide bonds; a recognition site for members of the proprotein convertase family of proteolytic enzymes, a consensus furin cleavage site (CFCS); and a C-terminal propeptide that includes a single-spanning transmembrane (TM) domain (Jovine et al., 2005; Monné et al., 2006; Bokhove et al., 2016; Wilburn & Swanson, 2017).

The mammalian fertilization process has been extensively described by both physiology and molecular biology. ZP and Juno oocyte proteins play a key role in this process, facilitating the interaction and fusion between the oocyte and the sperm respectively. Their interactions with sperm counterparts may give rise to a prezygotic reproductive isolation mechanism. Owing to protein specificity of these interactions, they are involved in the species-specific barrier (Florman & Ducibella, 2006).

These proteins are representative components involved in reproduction and are usually found evolving under positive selection. It is proposed that this favored rapid evolution plays an important role in reproductive isolation of diverging taxa (Swanson et al., 2001; Swanson & Vacquier, 2002; Turner & Hoekstra, 2008). Also, traits of coevolution between sperm and egg protein counterparts were reported to conduct reproductive isolation (Swanson et al., 2003; Aagaard et al., 2006). Up till now, sperm counterparts for oocyte gamete interaction proteins have not been identified in mammals; therefore, in this work we will specifically focus on egg coat protein positive selection marks in order to look into their role in speciation.

Our non-model species of study -the jaguar (*Panthera onca*)- is the most important predator in the South American bioma and in danger of extinction in Argentina (Paviolo et al., 2016). Hence, it is crucial to develop genomic, evolutionary and functional studies. Genomic introgression analysis carried out alongside with the first release of the jaguar complete genome exposed a complex pattern of historical inter-specific hybridization in the Pantherinae lineage (Figueiró et al., 2017). These findings revealed high rates of fertilization among pantherines, particularly between *Panthera leo* and *Panthera onca*, a fact that would indicate an absence of inter-specific isolation barriers. We aimed to

uncover the underlying molecular basis of this inter-species crossing and evolution of the functional capacities of pantherine ZP and JUNO proteins.

We hypothesize that sperm-oocyte interaction and fusion proteins are particularly conserved or accumulated few diversifying changes along the Pantherinae lineage, preventing prezygotic reproductive isolation.

Previous results that support our hypothesis are a), pervasive genealogical discordance across Panthera genomes, caused by both incomplete lineage sorting and complex patterns of historical interspecific hybridization (Li et al., 2016; Figueiró et al., 2017); b), natural hybrids between all panther species (Yadav et al., 2019); c), the high efficiency to obtain hybrids by IVF of oocytes with whole Zona Pellucida between different felines (Duque Rodriguez et al., 2021); and d), fast evolution of ZP genes could make them good candidates to contribute to prezygotic species isolation (Turner & Hoekstra, 2008., 2008, Meslin et al., 2012).

This work analyzes the genetic basis of the lack of prezygotic isolation among pantherine species through an evolutionary analysis of ZP1, ZP2, ZP3, ZP4 and IZUMO1R proteins in Carnivores, Feliformes, Caniformes and Pantherines.

2. Materials and Methods

2.1 Sequence retrieval and alignment

Jaguar sequences were obtained from the first version of the jaguar reference genome (Figueiró et al, 2017) and/or the HiC assembled jaguar genome (DNA zoo) by performing a local BLASTn using leopard (*Panthera pardus*) ortholog sequence as query, obtained from GenBank (NCBI Resource Coordinators, 2016). Other mammalian species sequences were obtained from Ensembl (Howe et al., 2021) and GenBank Nucleotide database (Supplementary tables 10, 11, 12, 13, 14). Visualization and multiple sequence alignments were performed with MEGA 5.2 (Kumar et al., 2018). Clustal and/or Muscle, as implemented in MEGA 5.2, were used for sequence alignments. A careful manual curation of the alignments was performed in order to maximize quality of aligned sequences. Low quality sequences that could not be replaced or significantly improved were discarded. Low quality or non-aligning portions of coding sequences in one species were deleted and, whenever possible, replaced using additional sequence sources such as Nscan and GeneScan, accessed through UCSC Genome Browser in order to guarantee homology of the aligned sequences.

2.2 Positive selection analysis

In order to detect signatures of molecular adaptation we used the modified Model A branch-site test 2 of positive selection (Zhang et al., 2005) applied in codeml program from the PAML4 package (Yang, 2007). This model has 2 variants: the focal branch variant and the subtree variant. In each case this model is able to detect signatures of positive selection in preassigned specific branches or clades respectively, and in both cases these changes can be assigned to individual codon positions. We applied both models for each of the tested groups in this work: Carnivora, Feliformia,

Caniformia, Pantherinae, *Panthera onca*. Test 2 of the Model A of codeml consists of two nested hypotheses that must be compared: in the alternative hypothesis, positive selection is allowed only in the selected branch or clade, but not in the background branches or clades; and in the null hypothesis no positive selection is allowed in any branch or clade of the tree. The heterogeneity among protein sites was handled by site classes with different omega values (ω=dN/dS). In this model three omegas were used: $\omega_0<1$, $\omega_1=1$ (fixed) and $\omega_2>1$. In the alternative hypotheses site classes with ω_2 were enabled in the foreground branch or clade allowing the presence of positive selection, but in the null hypotheses only site classes with $\omega 0$ and $\omega 1$ were enabled, restricting sites to purifying selection or neutral evolution only. The fit of the gene data (sequence alignment and tree) to these hypotheses was obtained by maximum likelihood (ML) and evaluated by a likelihood ratio test (LRT) in which a statistic calculated from twice the difference of the ML obtained was compared to a chi-square distribution (1 degree of freedom) to infer the significance between hypotheses.

Given that this test for positive selection detection is a very parameter-rich model, we implemented it by following an ad-hoc methodology designed to prevent local minimum solutions and maximize rerun results consistency. Instead of running all parameters optimization while evaluating the selection model, we pre-computed all branch lengths under the Goldman & Yang codon-based evolutionary model (Goldman & Yang, 1994) using M0 model, also known as one-ratio model, from PAML package. Thus the total number of parameters was split and only model-specific parameters were optimized during the positive selection analysis.

2.3 Tree selection

Two phylogenetic trees were reconstructed for each of the studied genes: a species tree that follows the canonical mammalian phylogeny (as depicted in https://www.ensembl.org/info/about/speciestree.html) and a gene tree reconstructed by ML from our sequence alignments data using the Jones-Taylor-Thornton (JTT) amino acid substitution model with partial deletion treatment for missing data (85% site coverage cutoff). Although tree topologies were defined in these ways, branch lengths of the trees used in the branch-site positive selection analyses were estimated using M0 (one ratio) model from PAML package (see above). All positive selection analyses for each gene were carried out using the species tree and the gene tree. The fitting of each of these phylogenies to the sequence data in the corresponding alignment was assessed based on the results of KH (Kishino & Hasegawa, 1989) and AU (unbiased) tests, performed with CONSEL v0.20 (Shimodaira & Hasegawa., 2001). When the species tree was rejected ($p<0.05$), only the results obtained from the gene tree were considered. When none of the phylogenies were rejected, both result sets were considered.

2.4 Positive selected sites

Positive selected sites were obtained by the Bayesian posterior analysis (BEB) implemented in codeml's branch-site specific positive selection test a posteri of the maximum likelihood estimates (Yang et al, 2005). As site-specific models are sensitive to codon parameters, we repeated each analysis 6 times using 2 different codon equilibrium frequencies estimation parameter variants (CodonFreq parameter in PAML). Sites reported correspond to those consistent with at least 3 replicates in each of the 2 CodonFreq variants applied ('codon table' and '3x4') with a p-value of at

least 0.7 in all reruns. For cases where neither the species tree nor the gene tree were rejected, all these criteria had to be met in both species tree and gene tree based analysis.

2.5 Sequence Identities

Mean sequence identities for our taxonomic groups of interest were obtained from pairwise sequence identities comparisons from each of our alignments using Sequence Identity And Similarity online tool (SIAS – http://imed.med.ucm.es/Tools/sias.html). Caniformes vs Feliformes mean identity comparison for each protein were performed through a two sample T-Test (Welch's T-test). Boxen plot graphs were drawn with Python 2.7 using the Seaborn library (version 0.9.1).

2.6 Predicted structure model of ZP proteins

The predicted structural models for jaguar ZP proteins were performed with the DeepMind AlphaFold2 (Jumper et al., 2021) as implemented in ColabFold (Mirdita *et al*., 2021) using 20 templates (phenix versión). The University of California in San Francisco (UCSF) Chimera v1.16 program (Pettersen et al, 2004) was used for visualization and the backbone-dependent rotamer library was used for structural interpretation (Shapovalov et al., 2011). N-glycosylation and O-glycosylation sites were obtained from the UniProt database (The UniProt Consortium, 2021) and converted to jaguar positions with our multiple sequence alignments. The Coulombic Surface Coloring calculates electrostatic potential according to Coulomb's law (coulombic surface coloring, −10 to +10 kcal/(mol*e), red to blue).

2.7 Protein domain identification

Protein domains and their corresponding positions in jaguar were defined using specific associated bibliography (Nishimura et al., 2019; Raj et al., 2017; Han et al., 2010; Moros-Nicolás et al., 2018a; Aydin et al., 2016) and our highly curated sequence alignments for position conversion along ortholog sequences. Start and end positions of ZP protein domains were later corrected based on the three-dimensional models and the observed and expected secondary structure of each domain: secondary structure prediction for each ZP protein was obtained using PDBsum web server (Laskowski et al., 1997), PSIPRED 4.0 (Jones, 1999) and DISOPRED3 (Jones et al., 2014) and contrasted with the canonical ZP hemi-domains topology (Bokhove et al., 2018) in order to check if obtained domains fulfilled the structural characteristic features in number and type of secondary structures and also if they displayed all disulfide-bonds described as necessary for each domain correct folding.
Lineal schematic representation showing ZP protein domains were based on Bokhove et al., 2018.

3. Results

The study of protein evolution has greatly changed thanks to the growing availability of complete genome sequences. For the jaguar, our no-model system of study, global protein evolution analysis is now possible owing to the availability of the jaguar genome draft (Figueiró et al., 2017). Furthermore, we have complemented and improved our evolutionary analyses of gamete interaction proteins using a second version of jaguar chromosome-resolved genome assembled by Hi-C long-range sequencing (Dudchenko et al., 2017). Our analysis starts with ZP isoforms directly involved in

sperm-recognition, ZP3 and ZP2, and continues with the analysis of structural cross-linker ZP proteins, ZP1 and ZP4; finally, we extended our results to IZUMO1R and added two control proteins: one under non-selection and another under previously reported positive selection in jaguar.

3.1 Jaguar ZP3

Jaguar *Zp3* gene sequence (reported in online supplementary material) was obtained performing a local BLASTn over the chromosome-resolution Hi-C genome assembly of *Panthera onca* (from DNA Zoo) using leopard (*Panthera pardus*) sequence (GeneID:109250568) as query. Our previous attempts to obtain this sequence from the first version of the jaguar genome failed, as only a partial and fragmented sequence could be retrieved. With the complete sequence, we could confirm that *Zp3* gene in the jaguar consists of 1272 nucleotides organized in 8 exons (Figure 1A, top) which matches with all panterine species orthologs and also in most sequenced felid species, with the only annotated exception of the puma (*Puma concolor*) ortholog, which displays an extra exon, 9 in total.

ZP3 protein presents the simplest domain composition among all members of the ZP protein family as it displays the basic structural elements common to all ZP proteins: a signal peptide, one complete zona pellucida module, divided into its N-terminal and C-terminal moieties (Figure 1A bottom), a furin cleavage site and an hydrophobic C-terminal trans-membrane domain (TMD), all necessary for their secretion and incorporation in the zona pellucida matrix.

The *Zp3* positive selection analysis was performed with the ortholog sequences from a total of 34 species presented in the tree shown in Figure 1B. KH and AU tests did not reject either species or gene ZP3 trees. Therefore, only results consistently obtained in both analyses were considered. *Zp3* evolutionary analysis displayed no differential adaptations for the pantherines when compared to its sister groups. In fact, there is no evidence of molecular adaptation in *Zp3* within the whole Felidae family. However, Felidae do display a confirmed signal of episodic adaptation at its basal branch (red line) (Figure 1B and supplementary table S1).

In contrast to the absence of signatures of positive selection among felids, subtree analysis revealed events of adaptive change for ZP3 in its sister group, Caniformia (brown highlighted area), and also for the whole Carnivora clade (light green highlighted area) (Figure 1B and supplementary table S1).

ZP3 positive selected amino acids were found at positions 26, 27, 28, 46 and 344 (Figures 1A-D, and supplementary table S1). Three sites were detected congruent with the episodic adaptation event identified in the basal branch of Feliformia: amino acid positions 26 W>A/T, 46 E>H and 344 S/L>M (red arrowheads in figure 1A and 1B). Amino acid positions related to the evidence of molecular adaptation in ZP3 within the subtree of the whole Carnivora order were: 26 W>T/A/P/S, 27 L/D/Q>E/G/W/P/S and 28 L/D>R/G/T (light green arrowheads), with the last two sites (27 L/D/Q>W/P/S and 28 L/D>R/G/T) also involved in events of molecular adaptation within Caniformia subtree (brown arroheads in figures 1A and 1B; supplementary table S1). It is interesting to note that, with the exception of position 344, which stands in the most extreme region of the active ZP3 protein, just before the furin cleavage site, all remaining positive selected sites detected for ZP3 are located in the N-terminal extreme, and most of them are located in the linking peptide between signal peptide and ZP-N domain. A single site 46 E>H is located in the N-terminal edge of ZP-N

domain (Figures 1A and 1E and supplementary table S1). Beyond that, the ZP module of ZP3 lacks positive selected sites for any branch or subtree analyzed.

ZP3 N-glycosylation sites, shown as bright yellow spheres in figure 1C, are located in the same facet of ZP-N domain and are likely involved in ZP polymerization (Monné et al., 2008). O-glycosylation sites, shown as bright green spheres in figure 1C, are clustered in the ZP module inter-domain linker and are structurally close to each other. No positive selected site detected for any of the basal branches or subtrees were found to be interacting with any of these glycosylation sites.

3.2 Jaguar ZP2

Jaguar *Zp2* gene complete sequence (reported in online supplementary material) was obtained using leopard orthologous sequence (GeneID: 109255525) as query for a BLASTn over the jaguar chromosome-resolution Hi-C genome assembly (from DNA Zoo). Jaguar *Zp2* gene consists of 2151 nucleotides organized in 18 coding exons (Figure 2A top) and their number, grouping and distribution along the locus is conserved among all sequenced felid species, with the exceptions of jaguarundi (*Puma yagouaroundi*) ortholog (GeneID:121019416) that displays 19 exons, and 2 alternative transcripts (XM_025916776.1 and XM_025916778.1) of the puma (*Puma concolor*) ortholog, that also display an extra exon.

Contrary to the minimalist ZP protein domain composition of ZP3, ZP2 protein exhibits the highest number of domains among all members of ZP protein family. Besides the characteristic ZP module (subdivided in its N-terminal and C-terminal domains) present in all ZP proteins, ZP2 contains 3 additional ZP-N domains located N-terminal from the complete ZP module, which are named ZP-N1 to ZP-N3 (Figure 2A bottom).

In order to search for positive selection signatures in the evolutionary history of ZP2, orthologs from 35 mammal species presenting a complete ZP2 public gene sequence were aligned and used to reconstruct the tree shown in Figure 2B. KH and AU tests from CONSEL failed to reject both the species and the gene trees reconstructed for ZP2. Therefore, both evolutionary contexts were considered and only consistent results in both scenarios were kept.

Similar to the results obtained for ZP3, ZP2 evolutionary analyses showed no signatures of selection among pantherines or even among felids. Once more, these analyses displayed signatures of positive selection among the sister group Caniformia (brown highlighted area) and for the whole Carnivora subtree (light green highlighted area) (Figure 2B and supplementary table S2). Nevertheless, and in contrast to the findings for ZP3, the felids basal branch did not present signatures of episodic adaptive change. On the other hand, jaguar ZP2 branch did present a positive selection signal associated to residues located at positions 231 Q/E>V and 713 M/T>I in the jaguar sequence (Figure 2A bottom, 2C and 2D). No consistent positive selected site could be robustly identified in correlation with the evolutionary signal established for Caniformia subtree. However, we could effectively reveal 3 changes related to the positive selection events in ZP2 among species of the Carnivora subtree: positions 180 Q/K/E>L/G/P and 181 M/T>R/K/P in ZP-N2 domain, and position 346 P/L/G>Q/R/M/V in ZP-N3 domain (Figure 2A bottom, 2D and supplementary table S2). All

positive selected sites are located in C-terminal from ZP2 sperm binding region and the ovastatine cleavage site and therefore could not affect sperm-binding function.

3.3 Jaguar ZP1

Jaguar *Zp1* complete sequence (reported in online supplementary material) was obtained through local BLASTn over the Illumina genome assembly (Figueiró et al., 2017) using leopard *Zp1* sequence as a query (GeneID:109246518). Jaguar *Zp1* gene comprises 1818 nucleotides organized in 12 exons (Figure 3A top) and follows the same gene structure in quantity, grouping and distribution of exons along the locus as all other sequenced felids.

Beyond the ZP protein common structural organization of domains, ZP1 displays a single ZP-N domain and a trefoil domain (Figure 3A), considered important for the cross-linking between ZP filaments (Greve and Wassarman, 1985; Nishimura et al., 2019).

Pseudogenization is much more common for *Zp1* than for any other *Zp* gene along placental mammal species. *Zp1* appears as a pseudogene in all Caninae species (dog, dingo and red fox), cattle (cow, pig, goat, sheep) and even in some cetacean (dolphin) and Pinniped species (Antarctic fur seal and Weddell Seal) (Goudet et al., 2008; Moros-Nicolás et al., 2018a). As these species are included in the species set we used for the analyses of the other members of the ZP protein family, our ZP1 species collection was strongly reduced. In order to keep a similar number of species for each evolutionary analysis, in the case of ZP1 we included other outgroup species, such as bats, Western European hedgehog, European shrew and star-nosed mole (Figure 3B). The resulting species tree was rejected by KH and AU CONSEL tests ($p<0.001$), but not the reconstructed gene tree ($p=0.999$). Therefore only results obtained from the evolutionary analysis based in the gene tree were considered.

Among the proteins studied in this work, ZP1 is the one that shows the largest number of positive selected sites: 12 sites (Figure 3A,B,C). As with the proteins studied so far, there are no adaptive changes in Pantherines for ZP1 and positive selection was detected in Carnivora (light green highlighted area) and Caniformia subtrees (brown highlighted area in figure 3B and supplementary table S3). However, contrary to previous results in ZP3 and ZP2, ZP1 does display signatures of positive selection in Feliformia subtrees (light yellow highlighted area). Moreover, positive selected sites for this protein do present in the ZP module. Positive selected sites 116 P>R/H, 356 F>V/I and 422 Q>D detected in the Feliformia subtree correspond to the ZP-N1, ZP-N and ZP-C protein domains, respectively (Figure 3A light yellow arrowheads). Most sites detected in the analysis of Caniformia subtree (Figure 3A brown arrowheads: 35 S>G/H, 147 T/A>M/V, 308 L>F, 371 P>W, 384 R>Q/H, 398 Y/F>H) and in the whole Carnivora subtree analysis (Figure 3A light green arrowheads: 146 P>R/H, 207 T>A/V, 285 L>V/M, 371 P>W, 384 R>Q/H, 398 Y/F>H and 422 Q>D/H) also correspond to those same protein domains, specially the ZP module that gathers an important cluster of positive selected sites. Position 384 R>Q/H is of particular interest as it is located in the internal hydrophobic patch (IHP), one of the two conserved hydrophobic motifs involved in ZP polimerization (Jovine et al., 2004).

3.4 Jaguar ZP4

Jaguar *Zp4* gene has 11 exons with a total length of 1707 nucleotides and encodes a 569 aa long

polypeptide. Its complete coding sequence (reported in online supplementary material) was obtained via local BLASTn over the Illumina jaguar genome assembly (Figueiró et al., 2017) using leopard *Zp4* sequence as query (GeneID:109248980).

Given that ZP4 and ZP1 are considered to be paralogous genes formed as a consequence of the duplication of a common ancestral gene, both keep the same domain structure and have a of 45 aa long trefoil domain and a ZP-N1 subdomain in their amino-terminal region (Figures 3A and 4A).

ZP4 alignment and tree were reconstructed with ortholog sequences from 34 species (Figure 4B). KH and AU tests from CONSEL rejected the species tree ($p=1x10^{-5}$). Therefore only results obtained from the evolutionary analyses based in the gene tree were considered.

Unlike the other three ZP proteins, ZP4 does not show adaptation signals in the subtrees of carnivores and caniformes. Instead, we found positive selection marks in the subtrees of feliformia and pantherines. ZP4 is the only protein studied in this work that does not follow our proposed hypothesis.

Regarding ZP4 positive selected sites, jaguar position 505 S/F>L is the only positive selected change detected for the subtree of Feliformes. This site is located beyond the furin cleavage site (positions 496-501); therefore, it is not present in the mature functional protein.

The other two detected sites: 309 T>S and 396 G>V/A correspond changes that took place among pantherine species and are located in the central region of ZP module, more specifically in ZP-N domain and ZP-C domain, respectively.

3.5 Jaguar IZUMO1R

JUNO protein, also known as IZUMO1R, is present at the cell surface of the oocyte and is the receptor of the sperm cell surface protein IZUMO1. The interaction between JUNO and IZUMO1 is essential for sperm to dock to the surface of an egg (Bianchi et al., 2014), a crucial recognition step prior to membrane fusion (Inoue et al., 2015). Although neither protein has been conclusively shown to be a fusogen, there is recent evidence that IZUMO1 may have fusogenic activity (Brukman et al., 2022).

Jaguar *Izumo1r* complete sequence (reported in online supplementary material) was obtained through local BLASTn over the Illumina genome assembly (Figueiró et al., 2017) and the chromosome-resolution Hi-C genome assembly (from DNA Zoo) using leopard ortholog coding sequence as query (GeneID:109267358). Jaguar *Izumo1r* gene is only 729 nucleotides in length and is composed of 4 coding exons and its locus structure, number and disposition of exons is well conserved along all available felid species.

Jaguar IZUMO1R/JUNO protein is 243aa long peptide that starts with a 19aa long signal peptide and contains a folate receptor domain between its positions 26 and 201.

No signatures of positive selection were detected along the IZUMO1R Carnivora clades either using the species or their gene trees. All tested branches and subtrees (Carnivora, Caniformia, Feliformia,

Pantherines and *Panthera onca*) rendered non-significant results for positive selection (supplementary table S5).

3.6 Sequence Identities

In order to learn if IZUMO1R lacks positive selection sites because it has been highly conserved and has thus accumulated few total changes or if this constraint is only restricted to positive selection changes, we performed an analysis of sequence divergence among our studied taxonomic groups assessing sequence identity. Looking more broadly at the whole range of amino acid changes accumulated for these proteins along Carnivora evolutionary history, we can appreciate that IZUMO1R acquired in most taxonimic groups a similar amount of change to those reported for ZP proteins (supplementary table S6). However, while all ZP proteins retained some amount of adaptive mutations, none of the conserved changes in IZUMO1R were positively selected. With only a few exceptions among all tested branches, groups, and proteins, the average identity values of IZUMO1R for the studied groups is not lower than those of ZP proteins (supplementary table S6).

It is worth noting that Feliformes and Caniformes sister groups show significant differences in average sequence identity for all studied proteins except ZP4, always evincing Caniformes as a more diverse group and Feliformes as the one that features less variability (Figure 5 and supplementary table S7).

Even though Pantherines display the highest average identity in each case, the higher percentage of identity observed in felids when compared to caniformes is not due to a pantherines effect exclusively, as non-pantherine felids always produce an average identity similar to those of the whole Feliformes group (Figure 5).

3.7 Control proteins: TPM3, a jaguar positively selected protein and ACTN1, a neutrally evolving protein.

To demonstrate the reliability of the method used to detect positive selection and inferring sites potentially subjected to selection, we performed likelihood ratio tests on control proteins, using *Tpm3* one of the positively jaguar specific selected genes previously reported (Figueiró el al., 2017). Results shown in supplementary table S8 shows a p-value=0.0006.

To demonstrate that the likelihood methods do not spuriously detect selection, we performed likelihood ratio tests for the conserved housekeeping *Actn1* gene (Holterhoff et al., 2019) (a negative control protein). Supplementary table S9 shows the negative expected results.

4. Discussion

There are two possible mechanisms of selection in gamete interaction protein evolution that could explain how key genes of reproduction could evolve rapidly to contribute to reproductive isolation. In one of them, positive selection may result from interactions between individuals of the same

species. Differences between sexual strategies that involve gamete interaction possibilities in finding optimal fertilization rates can lead to sexual conflict. This conflict may maintain either polymorphism or extend a co-evolutionary competition between egg and sperm proteins. This mechanism will not be approached in this study because the counterpart proteins of gamete interaction in sperm have not been defined in mammals. In the second case, selection may occur if there are hetero-specific interactions. When gametes from different species meet, resulting in hybrids that have reduced fitness, selection changes protein interactions, reducing hetero-specific fertilization. Consequently, this implies an economy of reproductive efforts (Turner & Hoekstra, 2008). While the latter is the traditional way in which hetero-specific hybridization is frequently considered, here we explore a less treated consequence of hybridization that may play a fundamental role for small population endangered species: the inter-specific hybridization as an enabler for introgression, a mechanism that can introduce novel adaptive variations, and quickly raise diversity and increase fitness (Adavoudi et al., 2021). To do so, we measured and compared amino acid diversity for gamete interaction proteins among different Carnivora taxa and delved into their evolutionary histories assessing selective forces influence and uncovering events of rapid evolution and positive selection.

Each jaguar *Zp* and *Izumo1r* gene shows a well-conserved gene structure among feline species. In terms of numbers of coding exons and their distribution and grouping along the locus, each of our studied genes presents a high consistency for all feline species, with the exception of the species of the genus Puma (*P. concolor* and *P. yagouaroundi*), which present a higher variability in this regard. When compared to all the other felid species gene structures, puma displays an extra exon in *Zp3* gene and in its *Zp2* alternative annotated transcript, while jaguarundi displays this extra exon only for *Zp2* gene.

The search for signatures of episodic adaptation at the basal branches of our taxonomic groups of interest (Carnivora, Feliformia, Caniformia and Pantherinae) revealed no punctual event of selection at any of these specific lineages for any of our studied proteins but one. The only exception was the basal branch of feliformes in ZP3, which did display a signature of episodic positive selection. This result obtained for ZP3 in the basal branch of Feliformia means that the detected adaptive changes (26 W>A/T, 46 E>H and 344 S/L>M) occurred early in the history of this suborder, after their split from Caniformia, and were fixed by selection. Taking into account that the first two sites (26 W>A/T & 46 E>H) are located in the amino terminal region of ZP3 and the fact that the changes took place in the early history of the Feliformes and were later fixed, these changes could be involved in the surge of a prezygotic isolating mechanism between this group and its sister group Caniformia. However, we can conclude that no subsequent adaptations took place later among felid species in *Zp3* gene, as no other molecular adaptation signals were detected in its subtree, evincing no contribution of ZP3 to isolation among felid species, including pantherines. The absence of this kind of episodic selection in any other of the basal branches tested indicate that, with the exception of Feliformes in ZP3, none of the other groups of interest acquired changes during their early evolutionary history in these proteins that were later fixed by means of adaptive evolution.

A different general scenario was revealed when searching for positive selection signals in these proteins within the subtrees of the same taxonomic groups. Signatures of molecular adaptation were

repeatedly identified within the subtrees of some of these phylogenetic groups, mainly in the Carnivora order and Caniformia suborder in ZP proteins. ZP3 and ZP2 displayed the same pattern of subtree evolution showing signatures of selection in Carnivora and Caniformia subtrees, but not in Feliformia or Pantherinae subtrees. ZP1 evolution deviated from this pattern as it also presented signatures of positive selection in the subtree of Feliformes, although the result for the subtree of pantherines was again negative. ZP4 is the subunit whose pattern of evolution differs more from the ones previously detailed, as it does not present signatures of selection among the subtrees of Carnivora or Caniformia. Moreover, Zp4 is the only gamete interaction protein studied here that shows evidence of diversifying positive selection in the subtree of Pantherinae as well as for the broader Feliformia suborder. Lastly, IZUMO1R shows no signature of diversifying positive selection in any of the basal branches or subtrees of the studied taxonomic groups.

Positive selected sites detected for ZP3 among Carnivora and Caniformia subtrees overlap and cluster with Feliformia basal branch related sites gathering in the amino terminal region, in particular the inter-domain peptide that links the signal peptide and ZP domain (Figure 1A bottom). As to positions with selective changes clustered in this portion of ZP3 protein, position 26 of the jaguar sequence is found as a tryptophan in most mammals but it varies among different Caniformia subgroups, particulary in Pinnipeds: 26 W>P/S. This is also one of the sites detected for the basal branch of Feliformes, along with position 46, and due to these selective changes in both clades it emerges as a positive selected site in the subtree of Caniformia. Regarding positions 26 and 27, they both show an increased selected diversity among Caniformia subgroups and thus appear as positive selected sites both in Caniformia and in Carnivora. Altogether, these results indicate that positive selected changes for ZP3 may have contributed to the specialization events that occurred inside Carniformia suborder or in the divergence between this group and Feliformia, but none of these changes would contribute to a prezygotic isolation of Panthera species or even between felid species.
A similar reasoning can be made for ZP2, as positive selection for this protein was detected inside Carnivora and Caniformia subtrees but not inside Feliformia or Pantherinae subtrees. However, ZP2 evolutionary analysis also displayed a signature of positive selection in the jaguar terminal branch. This species-specific adaptation of jaguar ZP2 could be traced to a single amino acid position: site 231 in the jaguar sequence, where a valine (V) replaces the highly conserved glutamine (Q) in nearly all mammals. The other 3 detected sites (180, 181 and 346) correspond to positions that were selected within the Carnivora subtree. Although a clear signature of adaptation was detected for the subtree of caniformia, no sites could be individually identified by BEB analysis. This could be explained by the combination of multiple restrictive criteria that we requested for a site to be unmistakably identified as positively selected in the final results. Residues 180 and 181, detected in Carnivora subtree analysis and residue 231, detected as positive selected exclusively in the jaguar branch, are all located in ZP-N2, the domain that contains ZP2 cleavage site from which gamete recognition in mice depends (Galhay et al., 2010; Avella et al., 2014; 2016). In the jaguar sequence the cleavage site appears between positions 171-172. All these sites are located in the C-terminal from the mentioned cleavage site and outside ZP2 sperm-binding region, which in the jaguar protein extends from position 55 to position 154. Therefore, the 3 positive selected sites detected in ZP-N2 domain of ZP2 would not compromise gamete recognition and sperm penetration through the zona pellucida.

Among all studied proteins, ZP1 was the one that showed a higher number of positive selected sites, 12 sites, compared to the rest of the ZP proteins that showed between 3 and 5. Although ZP1 and ZP4 remain by far the less studied components of the mammalian ZP, Jovine's lab structurally characterized ZP1 cross-linking function in human proteins (Monné & Jovine, 2011; Nishimura et al., 2019). Studies of human ZP1 I390fs404X mutant protein suggest that lack of a ZP in infertile patients carrying the corresponding mutation could be due to impaired secretion of ZP1 and the consequent absence of ZP filament cross-linking. As this step is prior to the cross linking with ZP3 it cannot discriminate how this region of ZP1 would interact with ZP3 if at all (Huang et al., 2014). On the other hand, the trefoil/ZP module region of ZP1 does not form non-covalent homodimers in humans, which shows that ZP1-N1 necessarily participates in cross-linking to maintain human ZP with its consequences over fertility (Nishimura et al., 2019).

Two other mutations were reported to negatively affect the cross-linking ability of human ZP1. One is the replacement of a residue of ZP1-N1 β- strand C with an additional Cys (R61C) (Yuan et al., 2019) or the truncation of the protein at the level of the ZP1-N1 bc loop (P54fs64X) (Sun et al., 2019). Then, there are two different regions to take into account in the crosslinking function of ZP1: one is the ZP1-N1 domain, in which we found two positive selective amino acids (35 inside Canifornia subtree and 116 in Feliformia) but both are outside the influence of the critical C66 region. The other important functional region is the ZP-C domain in which three aa were positively selected (384, 398 in Carnivora and Canifornia, and 422 in Carnivora and Felifornia). Although molecular basis of egg coat filament cross-linking by ZP1 is conserved between birds and mammals, it is less plausible that ZP1 could be involved in the species-specific isolation.

ZP4 was the last of the mammalian ZP proteins to be identified (Lefièvre et al., 2004) as it not present in the mouse (*Mus musculus*) the most extended and used mammalian model. Its coding gene is considered, along with *Zp1*, a consequence of a recent duplication event (Goudet et al., 2008) and both proteins conserve the same arrangement of domains, being the only two members of the ZP proteins family that display a trefoil domain. As a consequence, their cross-linking functions can be considered to be similar, overlapping or even redundant and this would be supported by the fact that the absence of any of these proteins in some species does not cause sterility (Goudet et al., 2008). However, more recent findings reveal that human ZP4 does not functionally complement ZP1 in infertile patients carrying ZP1 mutations (Huang et al., 2014). This could be explained by the fact that ZP4-N1 does not form a non-covalent homodimer in humans as it does in other vertebrates (Nishimura et al., 2019).

All in all, we have found that sperm-oocyte interaction and fusion-related proteins studied in this research lack the degree of diversification necessary to fix a prezygotic reproductive isolation barrier between pantherines. Regarding the evolutionary histories revealed for ZP proteins, it is interesting to note that *Zp3* and *Zp2*, genes that encode proteins directly involved in sperm recognition, display a similar pattern of adaptive evolution showing signatures of positive selection for Carnivora and Caniformia subtrees but not in Feliformia or Pantherinae subtrees. On the other hand, the evolution of *Zp1* and *Zp4*, the subunits involved in filaments cross-linking, does not concur with that pattern. Instead, they show signatures of positive selection for Feliformia in the case of *Zp1,* and Feliformia

and Panthera genus subtrees in the case of *Zp4*. Furthermore, this also extends to the location of positive selected sites along protein domains as both ZP1 and ZP4 display adaptive changes in their ZP domain while in ZP3 and ZP2 this domain is noticeably devoid of such changes.

These results are consistent with the previously reported pseudogenization events of *Zp1* in some carnivores, even-toed ungulates and cetacean species (Moros-Nicolás et al., 2018a; Goudet et al., 2008), the pseudogenization of *Zp4* in the house mouse (*Mus musculus*) (Lefièvre et al., 2004) and in two South American marsupials (common opossum and gray short-tailed opossum) (Moros-Nicolás et al., 2018b), the fact that *Zp4* appears to have diverged among vertebrates (Nishimura et al., 2019) and, finally, that ZP4 is not responsible for gamete interaction (Izquierdo-Rico et al, 2021). As *Zp4* and *Zp1* are recent paralogs that keep the same structure and domains, in those species where one of these cross-linking subunits has been pseudogenized the other would fulfill its function. Regarding *Izumo1r* evolution, selection analysis showed no signature of positive selection for any of the studied groups (Carnivora, Feliformia, Caniformia and Pantherines), either in their basal branches or inside their subtrees. These results strengthen the idea that ZP3 and ZP2 adaptive evolution contribute to the establishment of pre-zygotic barriers in species isolation while IZUMO1R, on the other hand, mediates the more conserved process of sperm docking to the egg wall.

Our evolutionary analyses revealed positive selection events in different groups along the history of Carnivora for every ZP protein but not for the fusion-related protein IZUMO1R. Selection analyses of the gene *Izumo1r* indicate that the driving evolutionary forces that ruled the history of this protein were purifying selection and neutral evolution. However, sequence identity results show that IZUMO1R did not remain immutable along mammalian evolution, but rather showed a similar number of changes as most ZP proteins in nearly all studied taxonomic groups. Sequence identity analyses of IZUMO1R and ZP proteins also revealed that Feliformia comprises low amino acid diversity when compared to its sister group, Caniformia, with the Pantherines exhibiting the lowest diversity. This difference between Caniformia and Feliformia was statistically significant in all but one of our studied proteins, ZP4, due to the fact that Caniformia has a comparatively higher degree of conservation in this protein.

The main limitation of this work lay in the difficulty of establishing a functional role for the positive selected sites. Although it is still widely believed that positive selection directly influences egg-sperm recognition, we do not exclude the possibility that positive selection may have in fact an indirect role. Experimental evidence comes from VERL/lysin complexes in which positive selection at the level of VERL repeat 1 (and to a lesser extent VERL repeat 2) generates a binding affinity gradient for lysin that results in species-specificity (Raj et al., 2017). However, this is not done by directly affecting residues at the interface. In fact, the divergence of repeat 1 may even be due to completely different reasons - for example pathogen avoidance (Raj et al., 2017).

All these results are consistent with previous evidence that shows ZP3 and ZP2 are essential for gamete recognition and, therefore conservation in these proteins would prevent the origin of a reproductive barrier between closely related species, allowing inter-species hybridization. This becomes more significant in the Panthera genus, characterized by species of big hypercarnivore

predators that occupy large home ranges, breed small litters and display low population densities. All these characteristics make these species more vulnerable to adverse environmental changes and especially to anthropogenic effects over their habitat that can cause an even further reduction in population size, fragmentation of the habitat and isolation of these small populations. All these effects will eventually bring to a decrease in genetic diversity of a population, a diminishing viability and a genomic erosion of the species, which could bring it to the edge of extinction (Abascal et al., 2016). One of the escape mechanisms from this fate is inter-species hybridization as a way to quickly increase genome diversity. As it is proposed, in species characterized by small population sizes, as is the case with pantherines, hybridization would pose an advantage to escape Allee effect and avoid extinction (Gómez-Sánchez et al., 2018). However, if key gamete interaction proteins have accumulated significant diversifying changes in these proteins between related species, cross-fertilization would be impossible. Conservation of these key proteins between such species would represent an advantage in challenging times and even save them from extinction. Moreover, the use of hybridization as a way to rapidly increase genetic diversity in a waning population or in reintroduction programs can serve as an effective tool in conservation efforts (Zecherle et al., 2021) and would, in some cases, even be useful to save a highly endangered species from extinction. This, of course, brings up an interesting debate in the field of species conservation as it transgresses a purist conception of a species identity and involves the concept of hybridization-induced-extinction (Todesco et al., 2016).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial o*r financial relationships that could be construed as a potential conflict of interest*.

Author Contributions

CC, CP and FP performed the bioinformatic analyses. FP designed the evolution study. CDB, FP, CC and CP performed jaguar ZP proteins structural models. TG contributes with phylogenic approaches and all concerning Vagalume jaguar genome data. PS conceived the project. FP and PS wrote the paper. All authors discussed the results and commented on the manuscript.

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Supplementary Material

Supplementary tables for this article can be found online attached to this manuscript.

Additional supplementary information will be reported online upon article publication or will be made available by the authors upon request.

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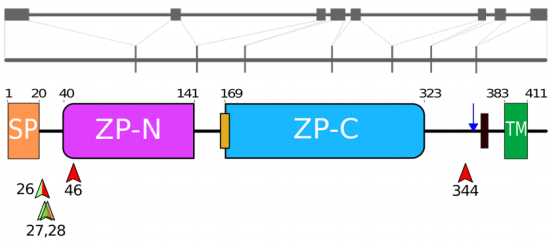
Figure 1

A



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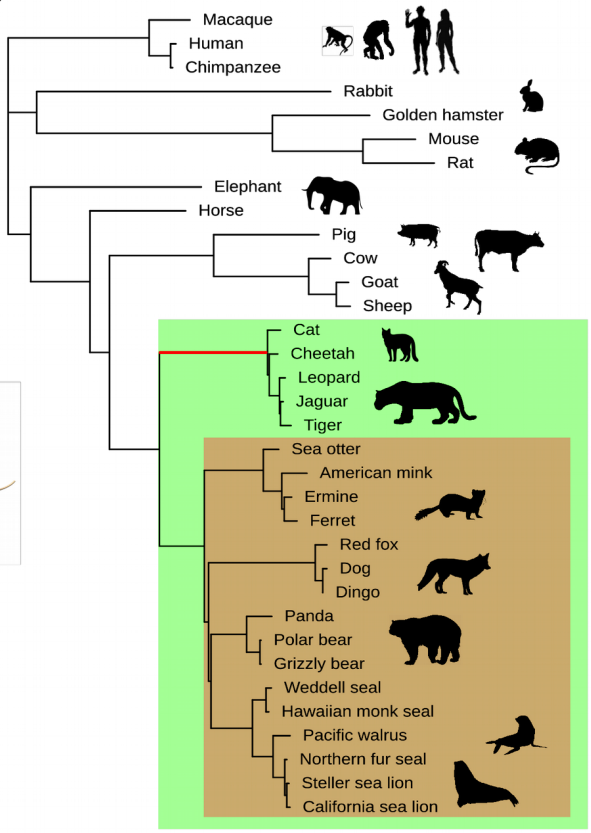

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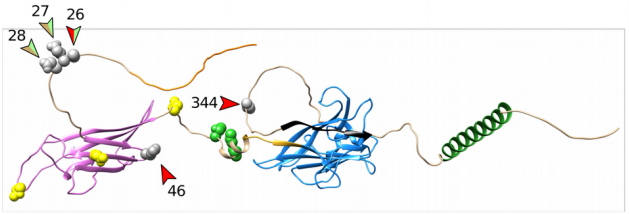


D

Position	Outgroups	Carnivora	
		Feliformia	Caniformia
26	W	T,A	P,S,W
27	L,D,Q	E,G	W,P,S
28	L,D	D	R,G,T
46	E	H	E
344	S,L	M	S

E

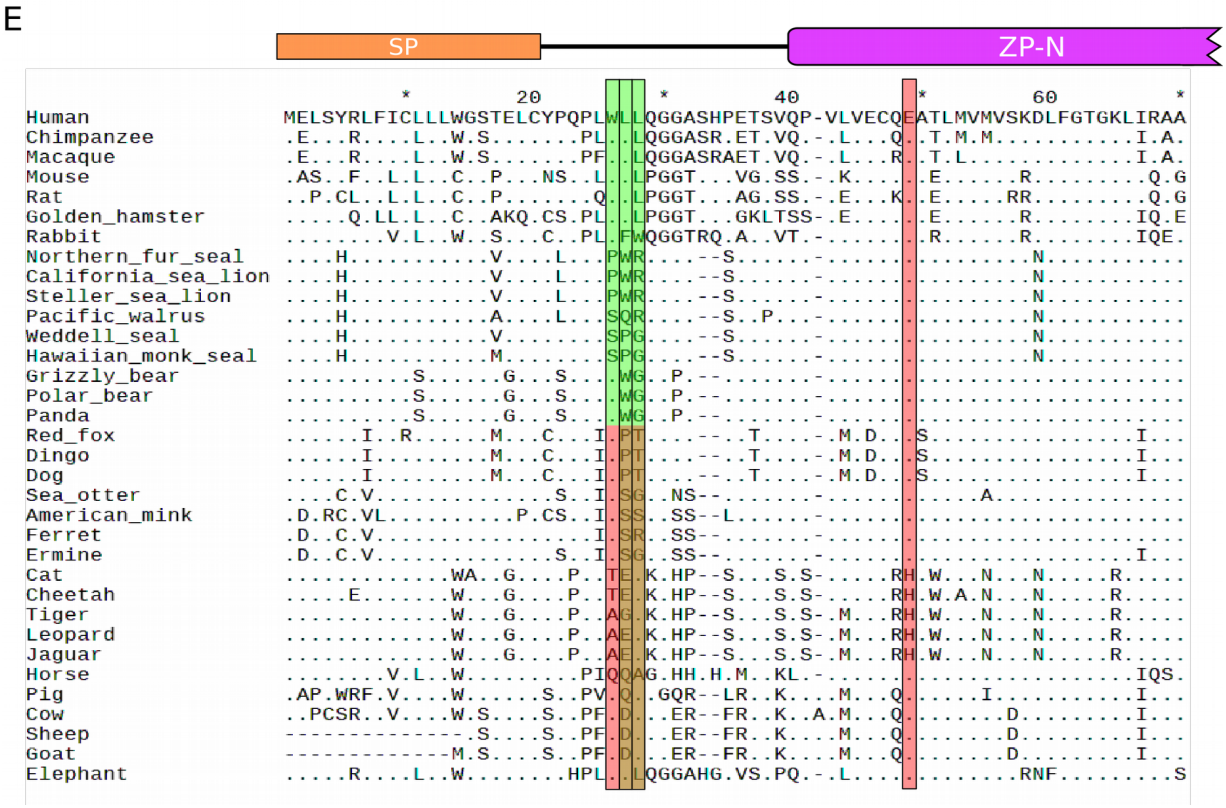
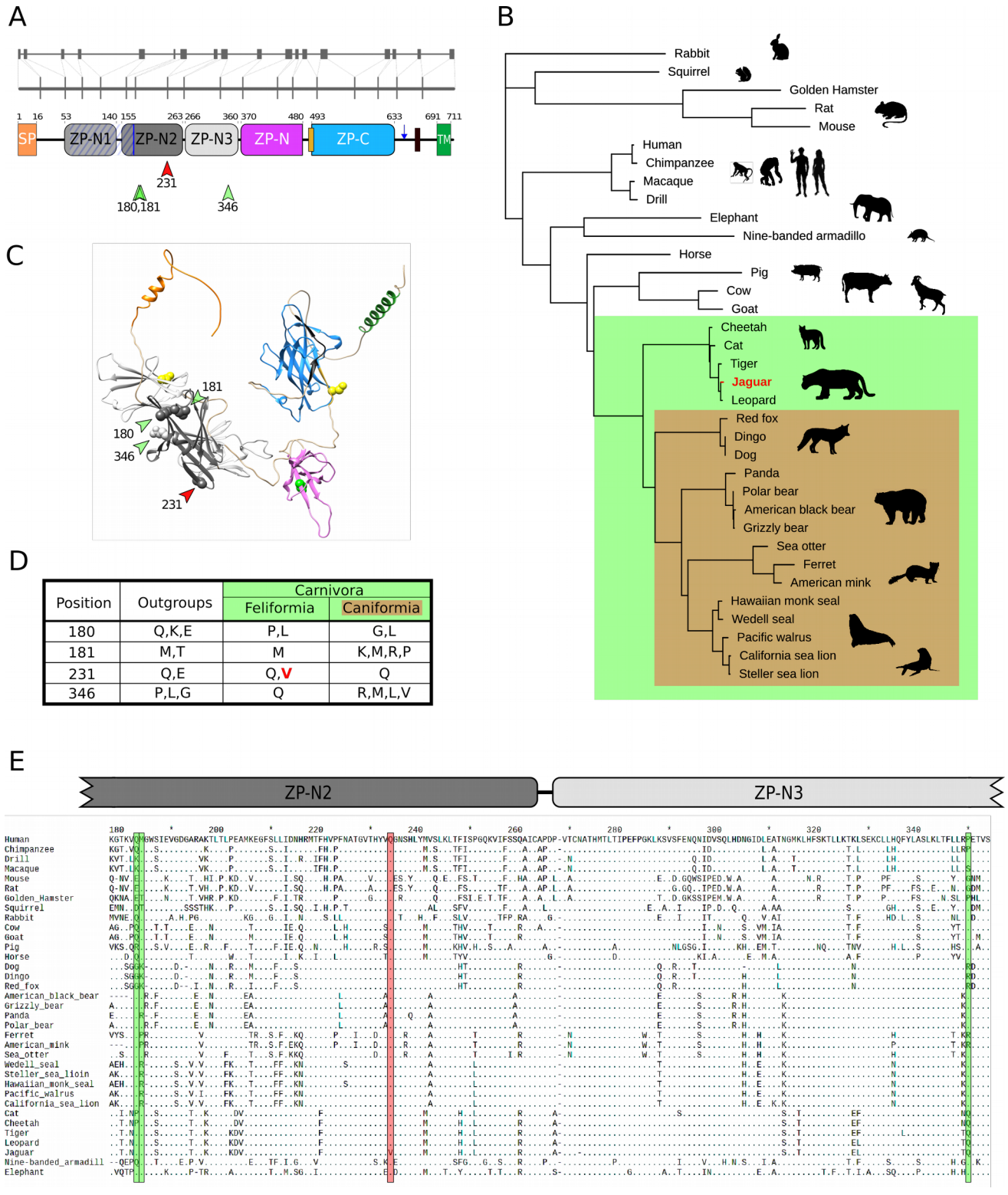


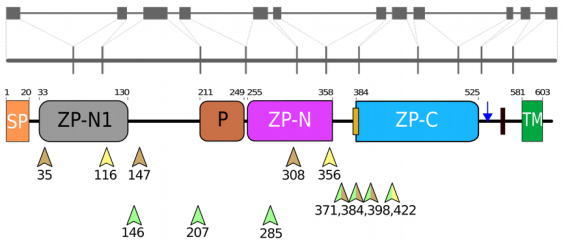
Figure 2



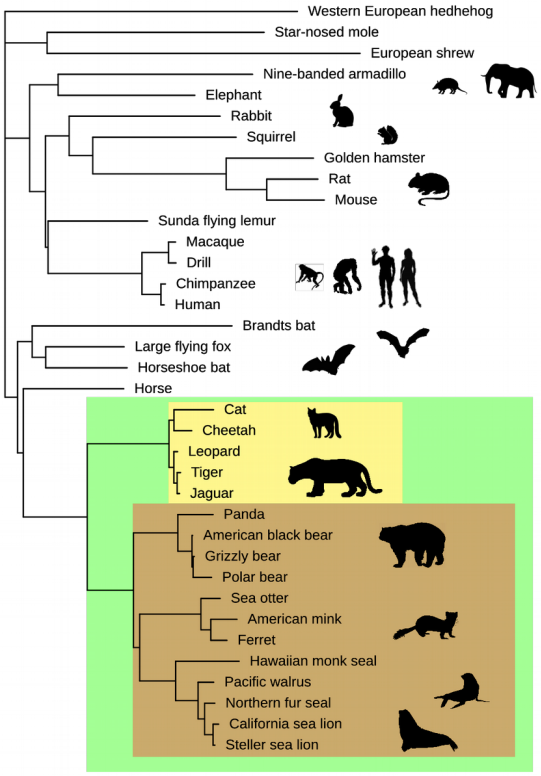
Position	Outgroups	Carnivora	
		Feliformia	Caniformia
180	Q,K,E	P,L	G,L
181	M,T	M	K,M,R,P
231	Q,E	Q,V	Q
346	P,L,G	Q	R,M,L,V

Figure 3

A



B



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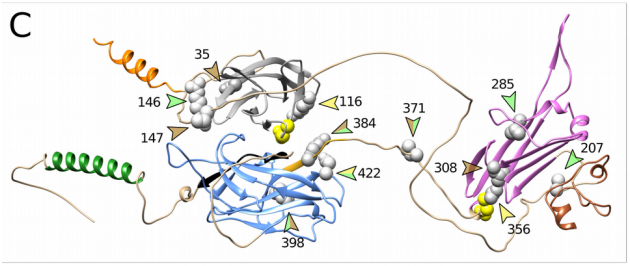
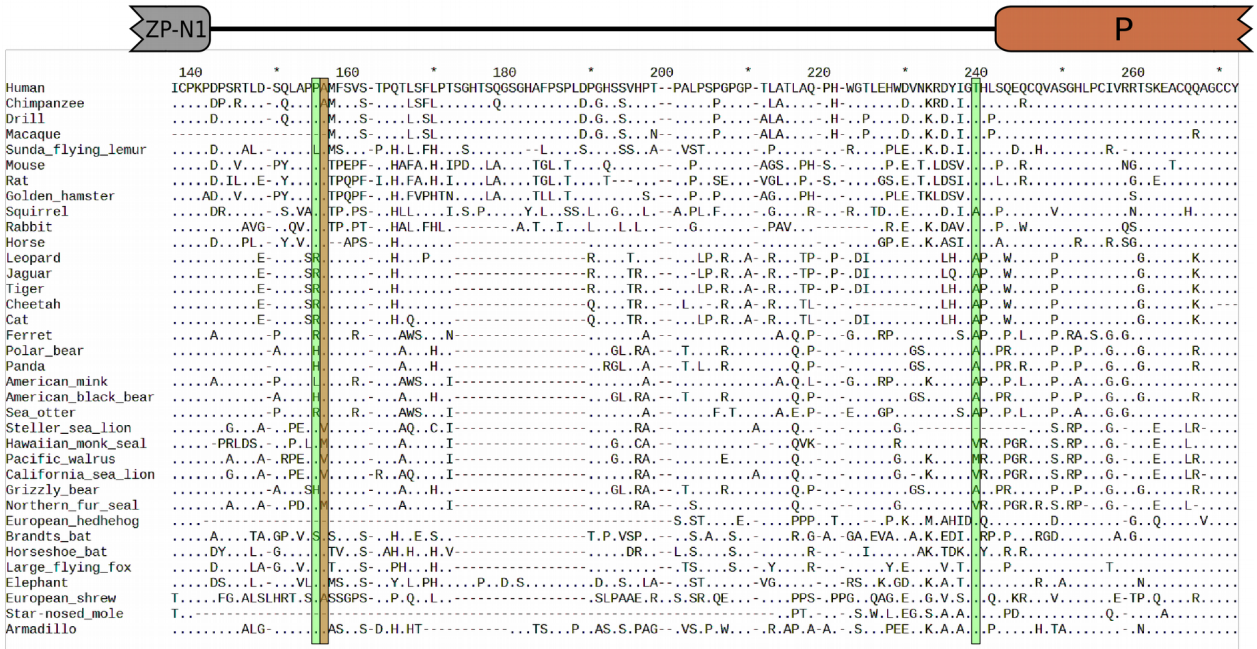


D

Position	Outgroups	Carnivora	
		Feliformia	Caniformia
35	S	S	G,H,S
116	P	R,H	P
146	P	R	R,H,P
147	T,A	T	M,V,T
207	T	A	A,V
285	L	V	M,L
308	L	L	F,L
356	F	V,I	F
371	P	P	P,W
384	R	R	Q,R,H
398	Y,F	F	Y,F,H
422	Q	D	Q,H

E



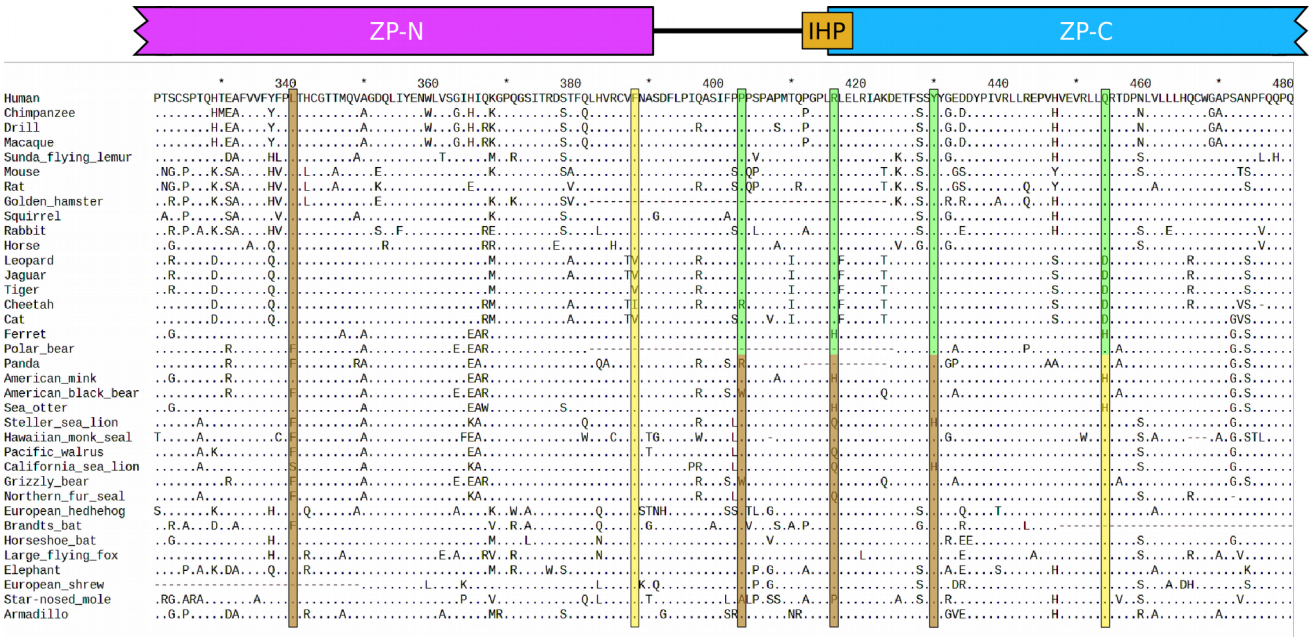
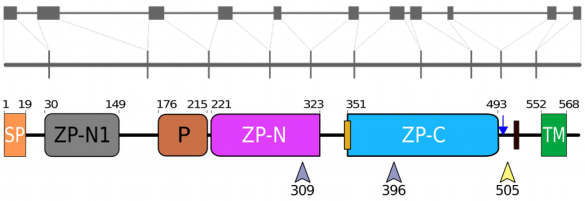

ZP-N
IHP
ZP-C
340
360
380
400
420
440
460
480
Human
Chimpanzee
Drill
Macaque
Sunda_flying_lemur
Mouse
Rat
Golden_hamster
Squirrel
Rabbit
Horse
Leopard
Jaguar
Tiger
Cheetah
Cat
Ferret
Polar_bear
Panda
American_mink
American_black_bear
Sea_otter
Steller_sea_lion
Hawaiian_monk_seal
Pacific_walrus
California_sea_lion
Grizzly_bear
Northern_fur_seal
European_hedhehog
Brandts_bat
Horseshoe_bat
Large_flying_fox
Elephant
European_shrew
Star-nosed_mole
Armadillo

Figure 4

A



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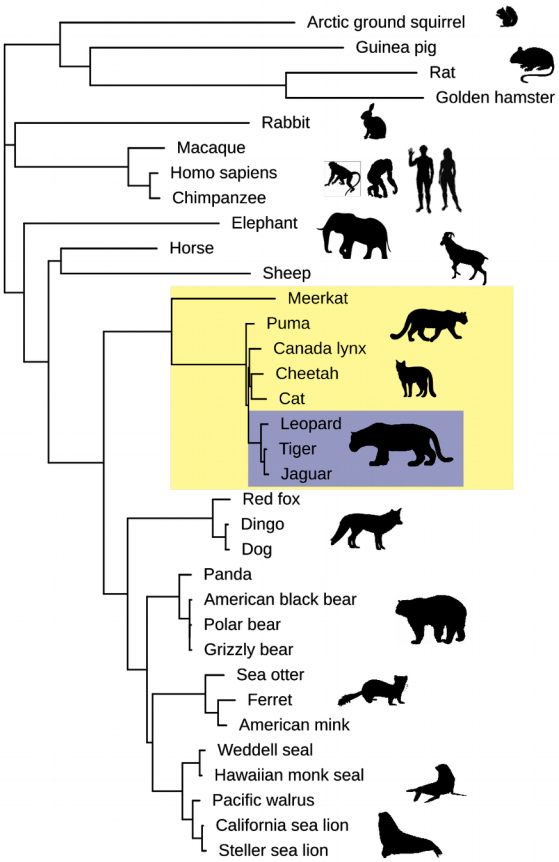

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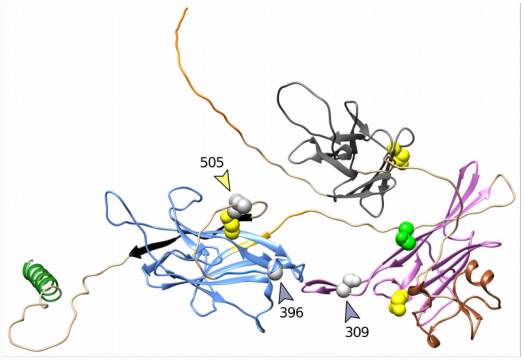

D

Position	Outgroups	Feliformia	Panthera
309	T	T	T,S
396	G,E	G	V,A
505	F,S	L,H	L

E

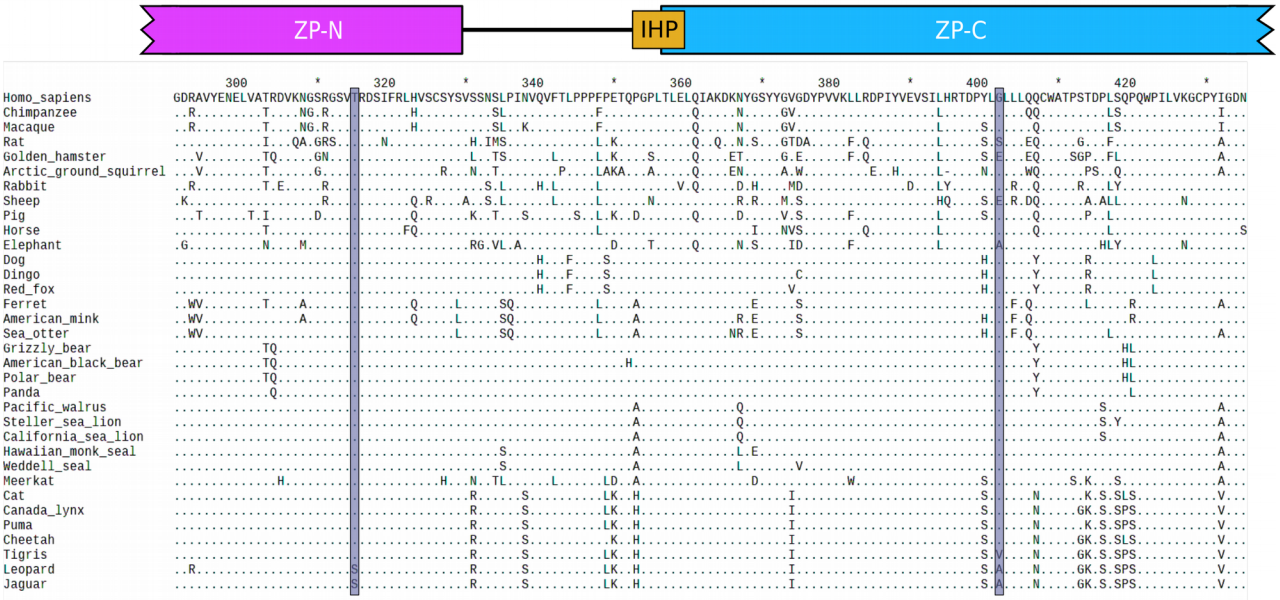

Figure 5

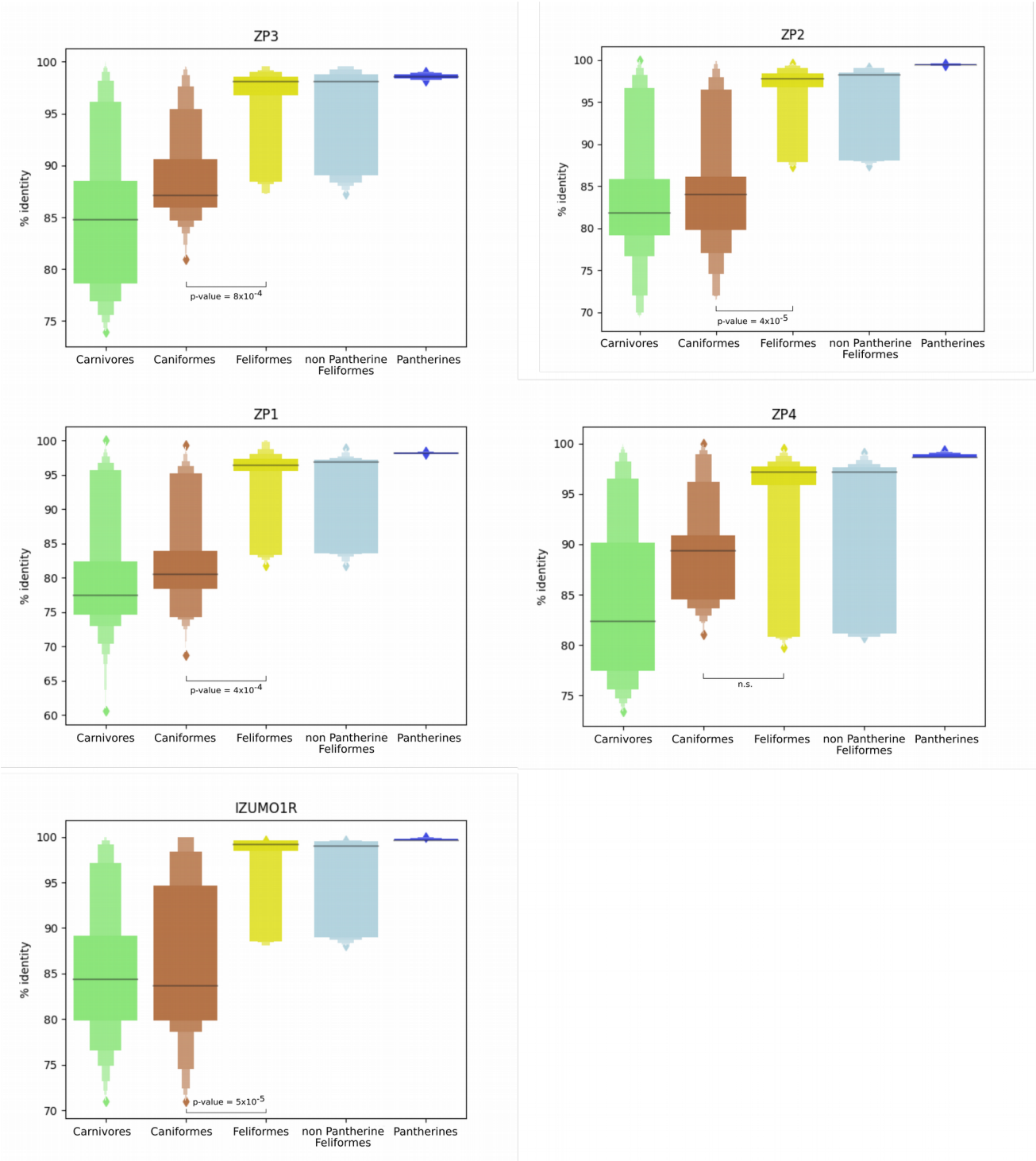
ZP3
% identity
p-value = 8x10^-4
Carnivores
Caniformes
Feliformes
non Pantherine Feliformes
Pantherines
ZP2
% identity
p-value = 4x10^-5
Carnivores
Caniformes
Feliformes
non Pantherine Feliformes
Pantherines
ZP1
% identity
p-value = 4x10^-4
Carnivores
Caniformes
Feliformes
non Pantherine Feliformes
Pantherines
ZP4
% identity
n.s.
Carnivores
Caniformes
Feliformes
non Pantherine Feliformes
Pantherines
IZUMO1R
% identity
p-value = 5x10^-5
Carnivores
Caniformes
Feliformes
non Pantherine Feliformes
Pantherines

Figure legends

1. Figure 1: ZP3 evolution. A. Jaguar *Zp3* gene schematic representation showing exons and introns (first line). Mature transcript of *Zp3* gene (second line). Start and end positions for each domain in the jaguar protein (third line) are displayed above and positive selected sites are indicated with arrowheads (fourth line) below the schematic representation of ZP3 protein domains. Red solid arrowheads indicate Feliformia basal branch positively selected sites. Split arrowheads with two colors denote sites that were detected in more that one analysis and their filling colors correspond to the highlighted subtrees or branches. SP: Signal Peptide; ZP-N: Zona Pellucida domain N-terminal moiety; ZP-C: Zona Pellucida domain C-terminal moiety; dark gray area: ZP3-specific subdomain; blue arrow pointing down: furin cleavage site; TM: transmembrane domain. B. Maximum likelihood ZP3 phylogenetic reconstruction for 34 species used in this analysis. Highlighted zones show positively selected Carnivora (light green) and Caniformia subtrees (brown). The basal branch of Feliformia suborder is colored in red to show the detection of positive episodic evolution along that branch. C. Three-dimensional model of the protein ZP3 shown in ribbons. N-glycosylated and O-glycosylated sites are shown as yellow and bright green atom spheres, respectively. Positive selected sites are indicated and shown as gray atom spheres. D. Positive selected sites table showing identity for each phylogenetic group. The identities in feline species of positive selected sites for Feliformes are shown in red. Autapomorphies in the background are not included. E. Detailed view of the first portion of ZP3 alignment showing the first 4 positive selected sites. The corresponding portion of the domain scheme is displayed above as a reference.

2. Figure 2: ZP2 evolution. A. Jaguar *Zp2* gene exons and introns model (first line). Mature transcript of *Zp2* gene (second line). Start and end positions for each domain in the jaguar protein (third line) are displayed above and positive selected sites are indicated with arrowheads (fourth line) below the schematic representation of ZP2 protein domains. The solid red arrowhead indicates the only positively selected sites detected for the jaguar lineage. Light green filled arrowheads indicate sites under selection in the Carnivora subtree. SP: Signal Peptide; ZP-N1/2/3: ZP-N like domains; diagonal striped blue pattern: ZP2 sperm-binding region; blue solid vertical line: ZP2 ovastatin cleavage site; ZP-N: Zona Pellucida domain N-terminal moiety; ZP-C: Zona Pellucida domain C-terminal moiety; blue arrow pointing down: furin cleavage site; green rectangle: transmembrane domain. B. ZP2 phylogeny reconstructed by maximum likelihood for 35 mammal species. Carnivora and Caniformia subtrees are featured in light green and brown respectively to indicate that signatures of selection were detected among them. Jaguar terminal branch is colored in red to show the detection of positive selection in that particular terminal branch. C. Three-dimensional model of the protein ZP2 shown in ribbons. N-glycosylated and O-glycosylated sites are shown as yellow and bright green atom spheres, respectively. Positive selected sites are indicated and shown as gray atom spheres. D. Table of positive selected sites detected for *Zp2* showing their position in the jaguar protein sequence and identity for each phylogenetic group. The identity in jaguar for the positive selected site in this species is shown in bold red. Autapomorphies in the background species are not included. E. Detailed view of the ZP-N2 and ZP-N3 portion of ZP2 alignment containing all 4 positive selected sites detected.

3. Figure 3: ZP1 evolution. A. Jaguar *Zp1* gene model showing 12 exons and 11 introns (first line). Mature transcript of *Zp1* gene (second line) aligned with the schematic representation of ZP1 protein. Start and end positions for each domain in the jaguar protein (third line) are displayed above and positive selected sites are indicated with arrowheads (fourth line) below the schematic representation of ZP1 protein domains. Below it, positive selected sites are indicated with colored arrowheads. Brown arrowheads correspond to positive selected sites inside Caniformia subtree. Yellow arrowheads correspond to positive selected sites for the Feliformia subtree. Light green arrows indicate sites under selection in the Carnivora subtree. Split arrowheads filled with two colors denote sites that were detected in more than one analysis and their filling colors correspond to the highlighted subtrees. SP: Signal Peptide; ZP-N1: ZP-N like domain; P: trefoil domain; ZP-N and ZP-C: Zona Pellucida domain N-terminal and C-terminal moieties; blue arrow pointing down: furin cleavage site; TM: transmembrane domain. B. ZP1 phylogeny reconstructed by maximum likelihood for 36 mammal species. Carnivora, Feliformia and Caniformia subtrees are featured in light green, yellow and brown respectively to indicate that signatures of selection were detected among them. C. Three-dimensional model of the protein ZP1 shown in ribbons. N-glycosylated sites are shown as yellow atom spheres. Positive selected sites are indicated and shown as gray atom spheres. D. Table of positive selected sites detected for *Zp1* showing their position in the jaguar protein sequence and their identity for each phylogenetic group of interest. Autapomorphies in the background species are not included. E1. Detailed view showing the interdomain region between ZP-N1 and trefoil domains of ZP1 alignment. E2. Detailed view for most of ZP1 ZP domain alignment. Positive selected sites in those regions are highlighted following the color code mentioned above. The Domain scheme is represented above each alignment.

4. Figure 4: ZP4 evolution. A. Jaguar *Zp4* gene model displaying its 11 exons and 10 introns (first line). Mature transcript of *Zp4* gene (second line) aligned with the schematic representation of ZP4 protein. Start and end positions for each domain in the jaguar protein (third line) are displayed above and positive selected sites among the Panthera genus are indicated with blue arrowheads (fourth line) below the schematic representation of ZP3 protein domains. SP: Signal Peptide; ZP-N1: ZP-N like domain; P: trefoil domain; ZP-N and ZP-C: Zona Pellucida domain N-terminal and C-terminal moieties; blue arrow pointing down: furin cleavage site; TM: transmembrane domain. B. Maximum likelihood phylogenetic reconstruction for ZP4 using 34 mammal species. Feliformia suborder and Panthera genus are highlighted in yellow and blue respectively to indicate that positive selection was detected in these subtrees. C. Three-dimensional model of the protein ZP4 shown in ribbons. N-glycosylated and O-glycosylated sites are shown as yellow and bright green atom spheres, respectively. Positive selected sites are indicated and shown as gray atom spheres. D. Table of positive selected sites detected for *Zp4* showing their position in the jaguar protein sequence and their identity for each phylogenetic group of interest. Autapomorphies in the background species are not included. E. ZP4 ZP-N and ZP-C domains alignments showing the 2 Panthera positive selected positions highlighted in blue. Domains corresponding to that area are aligned above.

5. Figure 5: ZPs and IZUMO1R sequence identities. Boxenplots graphs for ZP3, ZP2, ZP1, ZP4 and IZUMO1R showing mean sequence identity values (black horizontal line) for Carnivores (light green), Caniformes (brown), Feliformes (yellow), Pantherines (blue) and Feliformes species without

Pantherines (light blue). Caniformes vs Feliformes mean identity comparisons are significant for ZP3, ZP2, ZPp1 and IZUMO1R, but not for ZP4.

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Supplementary table S1: branch-site positive selection results for *Zp3*

	Log Likelihood	LRT	p	Parameters Estimation		
Carnivora						
Basal branch						
Alternative hypothesis	-11,311.2498	0.000	1.000	p0 = 0.6512	p1 = 0.3139	p2 = 0.0348
Null hypothesis	-11,311.2498			ω0 = 0.0960	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-11,305.8553	5.755	0.016	p0 = 0.6709	p1 = 0.3217	p2 = 0.0074
Null hypothesis	-11,308.7327			ω0 = 0.0962	ω1 = 1.0000	ω2 = 6.0157
Caniformia						
Basal branch						
Alternative hypothesis	-11,344.0445	0.000	1.000	p0 = 0.6746	p1 = 0.3253	p2 = 0.0000
Null hypothesis	-11,344.0445			ω0 = 0.0965	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-11307.45825	6.211	0.013	p0 = 0.6723	p1 = 0.3202	p2 = 0.0075
Null hypothesis	-11310.56391			ω0 = 0.0965	ω1 = 1.0000	ω2 = 6.4642
Feliformia						
Basal branch						
Alternative hypothesis	-11306.46675	5.815	0.016	p0 = 0.6702	p1 = 0.3214	p2 = 0.0083
Null hypothesis	-11309.3744			ω0 = 0.09489	ω1 = 1.0000	ω2 = 21.0815
Subtree analysis						
Alternative hypothesis	-11308.14697	1.973	0.160	p0 = 0.6582	p1 = 0.3177	p2 = 0.0250
Null hypothesis	-11309.1337			ω0 = 0.0933	ω1 = 1.0000	ω2 = 4.4878
Pantherinae						
Basal branch						
Alternative hypothesis	-11311.34381	0.000	1.000	p0 = 0.6746	p1 = 0.3253	p2 = 0.0000
Null hypothesis	-11311.34381			ω0 = 0.0965	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-11311.34381	0.000	1.000	p0 = 0.6746	p1 = 0.3253	p2 = 0.0000
Null hypothesis	-11311.34381			ω0 = 0.0965	ω1 = 1.0000	ω2 = 1.0000
Panthera onca						
Basal branch						
Alternative hypothesis	-11344.04454	0.000	1.000	p0 = 0.6746	p1 = 0.3253	p2 = 0.0000
Null hypothesis	-11344.04454			ω0 = 0.0965	ω1 = 1.0000	ω2 = 1.0000

LRT: statistical value of the likelihood ratio test; p0-2 and ω0-2 proportion of sites and dN/dS values estimated for each site class in the selection model (alternative hypothesis). respecively.

Supplementary table S2: branch-site positive selection results for *Zp2*

	Log Likelihood	LRT	p	Parameters Estimation		
Carnivora						
Basal branch						
Alternative hypothesis	-1.416.328.008	0.000	1.000	p0 = 0.5717	p1 = 0.4282	p2 = 0.0000
Null hypothesis	-1.416.328.008			ω0 = 0.1560	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-1.415.788.619	10.714	0.001	p0 = 0.5701	p1 = 0.4257	p2 = 0.0041
Null hypothesis	-1.416.324.311			ω0 = 0.1563	ω1 = 1.0000	ω2 = 7.4351
Caniformia						
Basal branch						
Alternative hypothesis	-19812.18614	0.000	1.000	p0 = 0.5559	p1 = 0.4440	p2 = 0.0000
Null hypothesis	-19812.18614			ω0 = 0.1719	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-19809.24815	5.865	0.015	p0 = 0.5565	p1 = 0.4410	p2 = 0.0023
Null hypothesis	-19812.18081			ω0 = 0.1729	ω1 = 1.0000	ω2 = 8.4665
Feliformia						
Basal branch						
Alternative hypothesis	-19812.18614	0.000	1.000	p0 = 0.5559	p1 = 0.4440	p2 = 0.0000
Null hypothesis	-19812.18614			ω0 = 0.1719	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-19812.18614	0.000	1.000	p0 = 0.5559	p1 = 0.4440	p2 = 0.0000
Null hypothesis	-19812.18614			ω0 = 0.1719	ω1 = 1.0000	ω2 = 1.0000
Pantherinae						
Basal branch						
Alternative hypothesis	-19812.18614	0.000	1.000	p0 = 0.5559	p1 = 0.4440	p2 = 0.0000
Null hypothesis	-19812.18614			ω0 = 0.1719	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-19812.18614	0.000	1.000	p0 = 0.5559	p1 = 0.4440	p2 = 0.0000
Null hypothesis	-19812.18614			ω0 = 0.1719	ω1 = 1.0000	ω2 = 1.0000
Panthera onca						
Basal branch						
Alternative hypothesis	-19807.08864	10.195	0.001	p0 = 0.5573	p1 = 0.4416	p2 = 0.0010
Null hypothesis	-19812.18614			ω0 = 0.1727	ω1 = 1.0000	ω2=999.0000

LRT: statistical value of the likelihood ratio test; p0-2 and ω0-2: proportion of sites and dN/dS values estimated for each site class in the selection model (alternative hypothesis). respecively.

Supplementary table S3: branch-site positive selection results for *Zp1*

	Log Likelihood	LRT	p	Parameters Estimation		
Carnivora						
Basal branch						
Alternative hypothesis	-22727.43086	0.000	1.000	p0 = 0.6149	p1 = 0.3850	p2 = 0.0000
Null hypothesis	-22727.43086			ω0 = 0.1542	ω1 = 1.0000	ω2= 1.0000
Subtree analysis						
Alternative hypothesis	-21711.1086	1993.045	<0.00001	p0 = 0.5189	p1 = 0.3224	p2 = 0.1586
Null hypothesis	-22707.6312			ω0 = 0.1418	ω1 = 1.0000	ω2= 1.0000
Caniformia						
Basal branch						
Alternative hypothesis	-22727.43086	0.000	1.000	p0 = 0.6149	p1 = 0.3850	p2 = 0.0000
Null hypothesis	-22727.43086			ω0 = 0.1542	ω1 = 1.0000	ω2= 1.0000
Subtree analysis						
Alternative hypothesis	-21715.84977	1998.271	<0.00001	p0 = 0.5065	p1 = 0.3123	p2 = 0.1811
Null hypothesis	-22714.98539			ω0 = 0.1461	ω1 = 1.0000	ω2= 1.0000
Feliformia						
Basal branch						
Alternative hypothesis	-21.694.68	0.112	0.738	p0 = 0.6110	p1=0.3644	p2 = 0.024
Null hypothesis	-21.694.74			ω0 =0.1600	ω1 =1.0000	ω2= 3.6648
Subtree analysis						
Alternative hypothesis	-21.686.82	12.992	3.13E-04	p0 = 0.6121	p1= 0.3485	p2 = 0.0393
Null hypothesis	-21.693.32			ω0 =0.1610	ω1 =1.0000	ω2 =6.5470
Pantherinae						
Basal branch						
Alternative hypothesis	-22476.2957	0.000	1.000	p0 = 0.6718	p1 = 0.3282	p2 = 0.0000
Null hypothesis	-22476.2957			ω0 = 0.1724	ω1 = 1.0000	ω2= 1.0000
Subtree analysis						
Alternative hypothesis	-22476.2957	0.000	1.000	p0 = 0.6718	p1 = 0.3282	p2 = 0.0000
Null hypothesis	-22476.2957			ω0 = 0.1724	ω1 = 1.0000	ω2= 1.0000
Panthera onca						
Basal branch						
Alternative hypothesis	-22476.2957	0.000	1.000	p0 = 0.6718	p1 = 0.3282	p2 = 0.0000
Null hypothesis	-22476.2957			ω0 = 0.1744	ω1 = 1.0000	ω2= 1.0000

LRT: statistical value of the likelihood ratio test; p0-2 and ω0-2 proportion of sites and dN/dS values estimated for each site class in the selection model (alternative hypothesis). respecively.

Supplementary table S4: branch-site positive selection results for *Zp4*

	Log Likelihood	LRT	p	Parameters Estimation		
Carnivora						
Basal branch						
Alternative hypothesis	-15035.12798	0.000	1.000	p0 = 0.5707	p1 = 0.4208	p2 = 0.0084
Null hypothesis	-15035.12798			ω0 = 0.1358	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-15033.84793	0.000	1.000	p0 = 0.5564	p1 = 0.3825	p2 = 0.0609
Null hypothesis	-15033.84793			ω0 = 0.1331	ω1 = 1.0000	ω2 = 1.0000
Caniformia						
Basal branch						
Alternative hypothesis	-15034.71063	0.843	0.359	p0 = 0.5738	p1 = 0.4213	p2 = 0.0047
Null hypothesis	-15035.13197			ω0 = 0.1358	ω1 = 1.0000	ω2 = 43.2308
Subtree analysis						
Alternative hypothesis	-15033.02388	0.000	1.000	p0 = 0.5332	p1 = 0.3836	p2 = 0.0832
Null hypothesis	-15033.02388			ω0 = 0.1289	ω1 = 1.0000	ω2 = 1.0000
Feliformia						
Basal branch						
Alternative hypothesis	-15035.13197	0.000	1.000	p0 = 0.5757	p1 = 0.4242	p2 = 0.0000
Null hypothesis	-15035.13197			ω0 = 0.1360	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-1,581,145,048	3.409	0.065	p0 = 0.5687	p1 = 0.4139	p2 = 0.0173
Null hypothesis	-1,581,315,528			ω0 = 0.1358	ω1 = 1.0000	ω2 = 3.7229
Pantherinae						
Basal branch						
Alternative hypothesis	-15035.13197	0.000	1.000	p0 = 0.5757	p1 = 0.4242	p2 = 0.0000
Null hypothesis	-15035.13197			ω0 = 0.1360	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-15021.54122	24.035	<0.00001	p0 = 0.5698	p1 = 0.4261	p2 = 0.0040
Null hypothesis	-15033.55865			ω0 = 0.1337	ω1 = 1.0000	ω2=129.468
Panthera onca						
Basal branch						
Alternative hypothesis	-15018.375955	0.000	1.000	p0 = 0.3736	p1 = 0.2793	p2 = 0.3471
Null hypothesis	-15018.375955			ω0 = 0.1358	ω1 = 1.0000	ω2=1.0000

LRT: statistical value of the likelihood ratio test; p0-2 and ω0-2 proportion of sites and dN/dS values estimated for each site class in the selection model (alternative hypothesis). respecively.

Supplementary table S5: branch-site positive selection results for *Izumo1r*

	Log Likelihood	LRT	p	Parameters Estimation		
Carnivora						
Basal branch						
Alternative hypothesis	-6617.15334	0.000	1.000	p0 = 0.7503	p1 = 0.2496	p2 = 0.0000
Null hypothesis	-6617.15334			ω0 = 0.1350	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-6610.17481	1.953	0.162	p0 = 0.7433	p1 = 0.2092	p2 = 0.0474
Null hypothesis	-6611.15111			ω0 = 0.1324	ω1 = 1.0000	ω2 = 1.7100
Caniformia						
Basal branch						
Alternative hypothesis	-6617.15334	0.000	1.000	p0 = 0.7503	p1 = 0.2496	p2 = 0.0000
Null hypothesis	-6617.15334			ω0 = 0.1350	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-6615.67307	0.000	1.000	p0 = 0.7048	p1 = 0.2298	p2 = 0.0653
Null hypothesis	-6615.67307			ω0 = 0.1292	ω1 = 1.0000	ω2 = 1.0000
Feliformia						
Basal branch						
Alternative hypothesis	-6617.15334	0.000	1.000	p0 = 0.7503	p1 = 0.2496	p2 = 0.0000
Null hypothesis	-6617.15334			ω0 = 0.1350	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-6617.15334	0.000	1.000	p0 = 0.7504	p1 = 0.2496	p2 = 0.0000
Null hypothesis	-6617.15334			ω0 = 0.1350	ω1 = 1.0000	ω2 = 1.0000
Pantherinae						
Basal branch						
Alternative hypothesis	-6616.64145	0.000	1.000	p0 = 0.3181	p1 = 0.1061	p2 = 0.5757
Null hypothesis	-6616.64145			ω0 = 0.1343	ω1 = 1.0000	ω2 = 1.0000
Subtree analysis						
Alternative hypothesis	-6617.15334	0.000	1.000	p0 = 0.7504	p1 = 0.2496	p2 = 0.0000
Null hypothesis	-6617.15334			ω0 = 0.1350	ω1 = 1.0000	ω2 = 1.0000
Panthera onca						
Basal branch						
Alternative hypothesis	-6617.15334	0.000	1.000	p0 = 0.7504	p1 = 0.2496	p2 = 0.0000
Null hypothesis	-6617.15334			ω0 = 0.1350	ω1 = 1.0000	ω2 = 1.0000

LRT: statistical value of the likelihood ratio test; p0-2 and ω0-2 proportion of sites and dN/dS values estimated for each site class in the selection model (alternative hypothesis). respecively.

Supplementary table S6: IZUMO1R - ZP proteins mean identity comparison for each taxonomic group of interest

Welch's T-Test p-value	IZUMO1R			
	Placental mammals	**Carnívora**	**Caniformia**	**Feliformia**
ZP3	0.665262	0.990030	0.107557	0.699292
ZP2	0.117486	0.395759	0.346810	0.596774
ZP1	0.00220862	0.0335943	0.291407	0.216625
ZP4	0.824827	0.716045	0.0667259	0.275288

p value < 0.05	Significant mean identity difference with IZUMO1R
p value > 0.05	Non-significant mean identity difference with IZUMO1R

Mean identity comparison for each protein were performed through a two sample T-Test (Welch's T-test).

Supplementary table S7: pairwise sequence identities report for taxonimic groups of interest In each studied protein

			Carnívora	Caniformia	Feliformia	Panterinae	Feliformia without pantherines	Otarridae	Placental mammals
ZP3	Identity	Mean	84.926	88.909	96.072	98.593	95.367	99.370	77.829
		Minimun	73.940	80.980	87.29	98.150	87.29	99.290	61.080
		Maximun	100.000	100.000	100	99.050	100	99.530	100.000
		Standar deviation	7.225	4.541	4.219	0.367	4.899	0.113	9.455
	Number of species		27	18	9	3	6	3	40
	p-value T-Test			0.0008					
					0.1127				
ZP2	Identity	Mean	83.158	84.764	95.770	99.440	94.935	99.440	73.378
		Minimun	69.310	71.420	87.300	99.300	87.440	99.160	53.820
		Maximun	100.000	100.000	99.580	99.580	99.160	99.860	99.860
		Standar deviation	7.552	7.137	4.317	0.114	5.003	0.302	11.200
	Number of species		27	18	9	3	6	3	42
	p-value T-Test			0.0000					
					0.0342				
ZP1	Identity	Mean	80.057	82.679	93.747	98.163	92.495	95.997	70.509
		Minimun	60.660	68.730	81.780	97.980	98.840	94.250	47.330
		Maximun	100.000	100.000	98.840	98.350	81.780	98.310	99.340
		Standar deviation	8.472	7.538	5.783	0.151	6.650	1.705	9.576
	Number of species		25	18	9	3	6	3	44
	p-value T-Test			0.0004					
					0.0513				
ZP4	Identity	Mean	84.134	89.538	93.735	98.823	92.034	99.360	76.445
		Minimun	73.410	81.080	79.750	98.590	80.700	99.360	58.770
		Maximun	100.000	100.000	99.470	99.290	99.120	99.360	99.640
		Standar deviation	7.994	5.053	7.020	0.330	7.873	0.000	10.162
	Number of species		27	18	9	3	6	2	38
	p-value T-Test			0.1443					
					0.0617				
IZUMO1R	Identity	Mean	84.901	85.402	96.887	99.720	95.802	98.633	76.930
		Minimun	70.980	70.980	88.06	99.580	88.06	98.36	55.830
		Maximun	100.000	100.000	100	100.000	100	99.18	100.000
		Standar deviation	7.405	7.706	4.461	0.197	5.024	0.386	9.280
	Number of species		27	18	9	3	6	3	42
	p-value T-Test			0.0001					
					0.0936				

Identity calculation were performed using Sequence Identity And Similarity online tool (SIAS – http://imed.med.ucm.es/Tools/sias.html). Caniformes vs Feliformes and Feliformes vs. Pantherines mean identity comparison for each protein were performed through a two sample T-Test (Welch's T-test).

Supplementary table S8: branch-site positive selection results for control proteins using *Tpm3*

Tpm3 (positive control)						
	Log Likelihood	**LRT**	**p**	**Parameters Estimation**		
Panthera onca						
Basal branch						
Alternative hypothesis	-2577.9999	7.53274	0.00606	p0 = 0.89229	p1 = 0.10658	p2 = 0.00113
Null hypothesis	-2581.76627			ω0 = 0.01625	ω1 = 1.00000	ω2 = 999.00000

LRT: statistical value of the likelihood ratio test; p0-2 and ω0-2 proportion of sites and dN/dS values estimated for each site class in the selection model (alternative hypothesis). respecively.

Supplementary table S9: branch-site positive selection results for control proteins using *Actn1*

Actn1 (negative contol)						
	Log Likelihood	**LRT**	**p**	**Parameters Estimation**		
Carnivora						
Basal branch						
Alternative hypothesis	-12050.00624	3E-05	1	p0 = 0.99388	p1 = 0.00612	p2 = 0.00000
Null hypothesis	-12050.00626			ω0 = 0.01547	ω1 = 1.00000	ω2 = 1.00000
Subtree analysis						
Alternative hypothesis	-12049.9043	-0.03271	1	p0 = 0.99262	p1 = 0.00598	p2 = 0.00139
Null hypothesis	-12049.88795			ω0 = 0.01525	ω1 = 1.00000	ω2 = 1.00000
Caniformia						
Basal branch						
Alternative hypothesis	-12050.00626	-3E-05	1	p0 = 0.99388	p1 = 0.00612	p2 = 0.00000
Null hypothesis	-12050.00624			ω0 = 0.01547	ω1 = 1.00000	ω2 = 1.00000
Subtree analysis						
Alternative hypothesis	-12048.75731	-0.0173	1	p0 = 0.99061	p1 = 0.00571	p2 = 0.00367
Null hypothesis	-12048.74866			ω0 = 0.01506	ω1 = 1.00000	ω2 = 1.00000
Feliformia						
Basal branch						
Alternative hypothesis	-12050.00624	0	1	p0 = 0.99388	p1 = 0.00612	p2 = 0.00000
Null hypothesis	-12050.00624			ω0 = 0.01547	ω1 = 1.00000	ω2 = 1.00000
Subtree analysis						
Alternative hypothesis	-12048.8892	0.4413	0.506496	p0 = 0.99227	p1 = 0.00617	p2 = 0.00156
Null hypothesis	-12049.10985			ω0 = 0.01527	ω1 = 1.00000	ω2 = 2.14145
Pantherinae						
Basal branch						
Alternative hypothesis	-12061.17908	-22.34567	1	p0 = 1.00000	p1 = 0.00000	p2 = 0.00000
Null hypothesis	-12050.00625			ω0 = 0.01701	ω1 = 1.00000	ω2 = 1.00000
Subtree analysis						
Alternative hypothesis	-12050.00624	0	1	p0 = 0.99388	p1 = 0.00612	p2 = 0.00000
Null hypothesis	-12050.00624			ω0 = 0.01547	ω1 = 1.00000	ω2 = 1.00000
Panthera onca						
Basal branch						
Alternative hypothesis	-12050.00799	-0.00348	1	p0 = 0.99388	p1 = 0.00612	p2 = 0.00000
Null hypothesis	-12050.00625			ω0 = 0.01546	ω1 = 1.00000	ω2 = 1.00000

LRT: statistical value of the likelihood ratio test; p0-2 and ω0-2 proportion of sites and dN/dS values estimated for each site class in the selection model (alternative hypothesis). respecively.

Supplementary table S10: ZP3 orthologs accession numbers

Species			Accession number
1	Hawaiian_monk_seal	*Neomonachus schauinslandi*	XM_021700471.1
2	Northern_fur_seal	*Callorhinus ursinus*	XM_025864635.1
3	Red_fox	*Vulpes vulpes*	XM_026019513.1
4	Grizzly_bear	*Ursus arctos horribilis*	XM_026516370.2
5	California_sea_lion	*Zalophus californianus*	XM_027612093.2
6	Steller_sea_lion	*Eumetopias jubatus*	XM_028110131.1
7	American_mink	*Neovison vison*	XM_044233042.1
8	Weddell_seal	*Leptonychotes weddellii*	XM_006733910.1
9	Pacific_walrus	*Odobenus rosmarus*	XM_004399076.2
10	Sea_otter	*Enhydra lutris*	XM_022501525.1
11	Polar_bear	*Ursus maritimus*	XM_008698535.2
12	Panda	*Ailuropoda melanoleuca*	XM_002925817.4
13	Dingo	*Canis lupus dingo*	XM_025426327.2
14	Dog	*Canis lupus familiaris*	NM_001003224.1
15	Ferret	*Mustela putorius furo*	NM_001310185.1
16	Chimpanzee	*Pan troglodytes*	XM_009453354.3
17	Macaque	*Macaca fascicularis*	XM_015447204.2
18	Tiger	*Panthera tigris*	XM_007073897.2
19	Leopard	*Panthera pardus*	XM_019421632.1
20	Cat	*Felis catus*	NM_001009330.2
21	Cocoy_Zp3-HiC_scaffold_9	*Panthera onca*	-
22	Cheetah	*Acinonyx jubatus*	XM_015078784.2
23	Ermine	*Mustela erminea*	XM_032326901.1
24	Rabbit	*Oryctolagus cuniculus*	NM_001195720.1
25	Rat	*Rattus norvegicus*	NM_053762.1
26	Golden_hamster	*Mesocricetus auratus*	NM_001281602.1
27	Mouse	*Mus musculus*	NM_011776.1
28	Human	*Homo sapiens*	NM_001110354.2
29	Elephant	*Loxodonta africana*	XM_003416567.2
30	Pig	*Sus scrofa*	NM_213893.1
31	Sheep	*Ovis aries*	XM_004020981.3
32	Cow	*Bos taurus*	NM_173974.3
33	Horse	*Equus caballus*	XM_001493044.3
34	Goat	*Capra hircus*	XM_018041042.1

Supplementary table S11: ZP2 orthologs accession numbers

Species			Accession number
1	Red_fox	*Vulpes vulpes*	XM_026003244.1
2	Weddell_seal	*Leptonychotes weddellii*	XM_006738322.2
3	Dog	*Canis lupus familiaris*	NM_001003304.2
4	Dingo	*Canis lupus dingo*	XM_035717942.1
5	Grizzly_bear	*Ursus arctos horribilis*	XM_044390073.1
6	Steller_sea_lion	*Eumetopias jubatus*	XM_028095252.1
7	Hawaiian_monk_seal	*Neomonachus schauinslandi*	XM_021692216.1
8	Sea_otter	*Enhydra lutris*	XM_022501447.1
9	American_mink	*Neovison vison*	XM_044233004.1
10	Pacific_walrus	*Odobenus rosmarus*	XM_004415752.1
11	American_black_bear	*Ursus americanus*	XM_045776421.1
12	California_sea_lion	*Zalophus californianus*	XM_027612146.1
13	Nine-banded_armadillo	*Dasypus novemcinctus*	XM_023558995.1
14	Elephant	*Loxodonta africana*	XM_023558995.1
15	Horse	*Equus caballus*	XM_001494769.4
16	Goat	*Capra hircus*	XM_005697589.3
17	Cow	*Bos taurus*	NM_173973.2
18	Pig	*Sus domesticus*	NM_213848.2
19	Polar_bear	*Ursus maritimus*	XM_008697192.2
20	Panda	*Ailuropoda melanoleuca*	XM_002927601.4
21	Ferret	*Mustela putorius furo*	XM_004780136.2
22	Drill	*Mandrillus leucophaeus*	XM_011976094.1
23	Macaque	*Macaca mulatta*	XM_015125771.2
24	Chimpanzee	*Pan troglodytes*	XM_016929619.2
25	Human	*Homo sapiens*	NM_001376232.1
26	Golden_hamster	*Mesocricetus auratus*	NM_001281671.1
27	Mouse	*Mus musculus*	NM_011775.7
28	Rat	*Rattus norvegicus*	NM_031150.1
29	Squirrel	*Ictidomys tridecemlineatus*	XM_040278570.1
30	Rabbit	*Oryctolagus cuniculus*	XM_017342508.1
31	Cat	*Felis catus*	NM_001009875.1
32	Leopard	*Panthera pardus*	XM_019430284.1
33	Tiger	*Panthera tigris*	XM_007084109.2
34	Cheetah	*Acinonyx jubatus*	XM_027042947.1
35	Jaguar	*Panthera onca*	-

Supplementary table S12: ZP1 orthologs accession numbers

Species		Accession number
1 Human	*Homo sapiens*	NM_207341.4
2 Chimpanzee	*Pan troglodytes*	XM_522022.6
3 Drill	*Mandrillus leucophaeus*	XM_011971090.1
4 Macaque	*Macaca mulatta*	XM_001084628.4
5 Sunda flying lemur	*Galeopterus variegatus*	XM_008585612.1
6 Mouse	*Mus musculus*	NM_009580.2
7 Rat	*Rattus norvegicus*	NM_053509.1
8 Golden_hamster	*Mesocricetus auratus*	NM_001281552.1
9 Squirrel	*Ictidomys tridecemlineatus*	XM_005331637.3
10 Rabbit	*Oryctolagus cuniculus*	NM_001270480.1
11 Horse	*Equus caballus*	XM_001493722.3
12 Leopard	*Panthera pardus*	XM_019414554.1
13 Jaguar	*Panthera onca*	-
14 Tiger	*Panthera tigris*	XM_007092010.3
15 Cheetah	*Acinonyx jubatus*	XM_027045024.1
16 Cat	*Felis catus*	NM_001284435.1
17 Ferret	*Mustela putorius furo*	XM_004770464.3
18 American_mink	*Neovison vison*	XM_044261161.1
19 Sea_otter	*Enhydra lutris*	XM_022507697.1
20 Polar_bear	*Ursus maritimus*	XM_040639172.1
21 Panda	*Ailuropoda melanoleuca*	XM_034645141.1
22 American_black_bear	*Ursus americanus*	XM_045786785.1
23 Grizzly_bear	*Ursus arctos horribilis*	XM_026487758.2
24 Steller_sea_lion	*Eumetopias jubatus*	XM_028118148.1
25 Hawaiian_monk_seal	*Neomonachus schauinslandi*	XM_021689794.1
26 Pacific_walrus	*Odobenus rosmarus*	XM_012565789.1
27 California_sea_lion	*Zalophus californianus*	XM_027580557.2
28 Northern_fur_seal	*Callorhinus ursinus*	XM_025878351.1
29 Western_european_hedhehog	*Erinaceus europaeus*	XM_007519649.1
30 Brandts_bat	*Myotis brandtii*	XM_014539193.1
31 Horseshoe_bat	*Rhinolophus ferrumequinun*	XM_033121271.1
32 Large_flying_fox	*Pteropus vampyrus*	XM_023522888.1
33 Elephant	*Loxodonta africana*	XM_003419500.2
34 European_shrew	*Sorex araneus*	XM_012935534.1
35 Star-nosed_mole	*Condylura cristata*	XM_012725157.1
36 Nine-banded_armadillo	*Dasypus novemcinctus*	XM_004446490.2

Supplementary table S13: ZP4 orthologs accession numbers

Species		Accession number
1 Human	*Homo sapiens*	NM_021186.5
2 Chimpanzee	*Pan troglodytes*	XM_024352844.1
3 Macaque	*Macaca nemestrina*	XM_011729442.2
4 Rat	*Rattus norvegicus*	NM_172330.3
5 Golden_hamster	*Mesocricetus auratus*	NM_001281648.1
6 Arctic_ground_squirrel	*Urocitellus parryii*	XM_026413538.1
7 Rabbit	*Oryctolagus cuniculus*	NM_001082295.2
8 Sheep	*Ovis orientalis aries*	XM_027962220.2
9 Pig	*Sus scrofa*	NM_214045.1
10 Elephant	*Loxodonta africana*	XM_023549132.1
11 Horse	*Equus caballus*	XM_001490753.4
12 Dog	*Canis lupus familiaris*	XM_038487243.1
13 Dingo	*Canis lupus dingo*	XM_025448645.2
14 Red_fox	*Vulpes vulpes*	XM_026008664.1
15 Sea_otter	*Enhydra lutris*	XM_022500198.1
16 Grizzly_bear	*Ursus arctos horribilis*	XM_026503989.2
17 Polar_bear	*Ursus maritimus*	XM_040624263.1
18 American_black_bear	*Ursus americanus*	XM_045802298.1
19 Panda	*Ailuropoda melanoleuca*	XM_019804231.2
20 American_mink	*Neovison vison*	XM_044236554.1
21 Ferret	*Mustela putorius furo*	XM_045087341.1
22 Pacific_walrus	*Odobenus rosmarus*	XM_004411337.1
23 Steller_sea_lion	*Eumetopias jubatus*	XM_028104607.1
24 California_sea_lion	*Zalophus californianus*	XM_027596652.1
25 Hawaiian_monk_seal	*Neomonachus schauinslandi*	XM_021696173.2
26 Weddell_seal	*Leptonychotes weddellii*	XM_006732888.1
27 Meerkat	*Suricata suricatta*	XM_029915014.1
28 Cat	*Felis catus*	NM_001009260.1
29 Cheetah	*Acinonyx jubatus*	XM_015085463.2
30 Puma	*Puma concolor*	XM_025915060.1
31 Canada_lynx	*Lynx canadensis*	XM_030335447.1
32 Tiger	*Panthera tigris*	XM_007086048.2
33 Leopard	*Panthera pardus*	XM_019419088.1
34 Jaguar	*Panthera onca*	-

Supplementary table S14: IZUMO1R orthologs accession numbers

Species			Accession number
1	Red_fox	*Vulpes vulpes*	XM_025993322.1
2	Dog	*Canis lupus familiaris*	XM_038429635.1
3	Dingo	*Canis lupus dingo*	XM_025419176.2
4	Grizzly_bear	*Ursus arctos horribilis*	XM_044386536.1
5	Panda	*Ailuropoda melanoleuca*	XM_002930414.3
6	Polar_bear	*Ursus maritimus*	XM_040630688.1
7	American_mink	*Neovison vison*	XM_044260702.1
8	Northern_fur_seal	*Callorhinus ursinus*	XM_025893961.1
9	Sea_otter	*Enhydra lutris*	XM_022514185.1
10	Steller_sea_lion	*Eumetopias jubatus*	XM_028119634.1
11	Ferret	*Mustela putorius furo*	XM_004754509.3
12	Hawaiian_monk_seal	*Neomonachus schauinslandi*	XM_021679101.1
13	Weddell_seal	*Leptonychotes weddellii*	XM_031036823.1
14	Pacific_walrus	*Odobenus rosmarus*	XM_004410756.1
15	California_sea_lion	*Zalophus californianus*	XM_027580383.2
16	Mouse	*Mus musculus*	NM_022888.3
17	Rat	*Rattus norvegicus*	NM_001398801.1
18	Golden_hamster	*Mesocricetus auratus*	XM_005084043.3
19	Squirrel	*Ictidomys tridecemlineatus*	XM_005337189.2
20	Rabbit	*Oryctolagus cuniculus*	XM_017349286.1
21	Pig	*Sus scrofa*	XM_013979283.2
22	Nine-banded_armadillo	*Dasypus novemcinctus*	XM_004471908.1
23	Horse	*Equus ferus caballus*	XM_014741864.2
24	Cow	*Bos taurus*	XM_024975624.1
25	Sheep	*Ovis orientalis aries*	XM_027979102.2
26	Human	*Homo sapiens*	NM_001393610.1
27	Chimpanzee	*Pan troglodytes*	XM_016922442.1
28	Drill	*Mandrillus leucophaeus*	XM_011995022.1
29	Macaque	*Macaca mulatta*	NM_001193805.1
30	Jaguar	*Panthera onca*	-
31	Leopard	*Panthera pardus*	XM_019450101.1
32	Tiger	*Panthera tigris*	XM_042958585.1
33	Cheetah	*Acinonyx jubatus*	XM_027039662.1
34	Cat	*Felis catus*	XM_023238987.2
35	Elephant	*Loxodonta africana*	XM_010595475.2