Evolution of Fertilization-Related Genes Provides Insights Into Reproductive Health in Natural Ascrotal Mammals

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Cryptorchidism is the failure of one or both testes to descend into the bottom of the scrotum. This common congenital malformation in humans and domestic animals is the best characterized risk factor for abnormal sperm functions and infertility. However, current treatment approaches for cryptorchidism do not ensure paternity in all cases. Some lineages of mammals (such as elephants and cetaceans) have natural ascrotal testes (i.e., undescended or incompletely descended testes) and normal sperm motility and fertility, providing an opportunity to understand the genetic basis of cryptorchidism. In this study, we showed that genes associated with sperm motility and competition/fertility in ascrotal mammals experienced frequent, strong selective pressure. The fixation of specific amino acids and positive selection in ascrotal mammals could affect the physicochemical properties and functions of fertilization-related proteins. In a comparison between mammals with undescended testes and incompletely descended testes, discrepancies in genes showing evidence for adaptive evolution and in functional enrichment suggested that multiple molecular mechanisms contribute to the maintenance of fertility in the challenging testicular environment. Our findings revealed substantial heterogeneity in the divergence of fertilization-related genes between natural scrotal and ascrotal mammals and provide insight into molecular mechanisms underlying normal sperm motility and competition in natural ascrotal mammals. We provide a detailed theoretical basis for understanding the pathology of cryptorchidism from a molecular evolutionary perspective. This study may contribute to the establishment of diagnostic and therapeutic targets for sperm motility and fertility disorders due to congenital cryptorchidism in humans and domestic animals.

Keywords: cryptorchidism, ascrotal testis, sperm motility, fertilization, adaptive evolution

INTRODUCTION

Cryptorchidism is the failure of one or both testes to descend into the bottom of the scrotum (Foresta et al., 2008). The cryptorchid testis is located in the intra-abdominal, inguinal, or high scrotal position. In newborn males, cryptorchidism is one of the most common congenital malformations, with a prevalence of 1.8–8.4% (Rodprasert et al., 2020). Moreover, cryptorchidism

GRAPHICAL ABSTRACT | Evolution of fertilization-related genes provides insights into reproductive health in natural ascrotal mammals. The fixation of specific amino acids and selection on sperm motility and competition-related genes contributed to normal fertility in ascrotal mammals. The silhouettes are reproduced from FLATICON (https://www.flaticon.com/).

is common in domestic mammals (e.g., horses, dogs, cattle, and sheep). For instance, it has been estimated that cryptorchidism affects 5–8% of male foals (Murase et al., 2020).

It should be noted that cryptorchidism is a well-characterized risk factor for abnormal sperm functions, poor semen quality, infertility, and testicular germ cell tumors in men (Foresta et al., 2008). A local high temperature in the testis and epididymis is a major risk factor for cryptorchidism (Ahmad et al., 2012). Heat stress damages the sperm chromatin structure, motility, competence for fertilization, and other properties (Sailer et al., 1997). In particular, as a common feature of spermatozoa, motility after capacitation is essential for fertilization (Holt and Van Look, 2004). The importance of sperm motility is highlighted by the fact that the measurement of motility forms an indispensable part of most semen or sperm evaluation criteria (Mocé and Graham, 2008). Generally, sperm motility parameters measured by the Computer-Assisted Semen Analysis (CASA) system [including curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), linearity (LIN), amplitude of lateral head displacement (ALH), straightness (STR), wobble (WOB), beat cross frequency (BCF), and total motility] are used to assess sperm quality in an objective and detailed manner. Investigating the biological relevance of CASA parameters in the context of prediction of male fertility potential has been important to understand fertilization and also for diagnosis and treatment of male infertility (Larsen et al., 2000).

Functionally defective sperm is the most frequent single defined cause of human infertility (Aitken and Curry, 2011; Gonçalves et al., 2021). However, typical early treatment of congenital cryptorchidism (i.e., hormonal and operative treatment) in scrotal species (including humans) does not ensure normal fertility and paternity or significantly reduce the risk of testicular cancer (Hutson et al., 1992; Rodprasert et al., 2020).

The etiology of cryptorchidism remains an enigma, and the identification of additional diagnostic and prognostic markers is needed.

Most adult male mammals have completely descended testes (CDT) from the position of primitive gonad into the scrotum (such as dogs, tigers, mice, and gorillas) (Kleisner et al., 2010; Hafez, 2012). However, some lineages of mammals have natural healthy ascrotal testes located in an intra-abdominal position or inside the inguinal region (Hutson et al., 1992). For example, monotremes (such as the platypus and echidna) and elephants have intra-abdominal testes positioned close to the kidney, called undescended testes (UDT). Cetaceans have incompletely descended testes (IDT) located lateral to the bladder neck in the inguinal region. It is the fact that ascrotal testes provide ecological and physiological benefits for some mammals. For example, as a consequence of axial style swimming in an aquatic environment, testes and epididymides located in inguinal region of male cetaceans and true seals benefits the streamlining body shape and reduces the energetic costs (Boice et al., 1964; Arkowitz and Rommel, 1985; Rommel et al., 2007). Notably, there are few records of sperm dysfunction in natural ascrotal mammals. Although the complex hormonal, anatomical, and environmental factors leading to cryptorchidism have recently been described (Barthold et al., 2008; Foresta et al., 2008; Chung and Brock, 2011), the molecular mechanisms underlying normal sperm functions in natural ascrotal mammals remain unexplored.

In the present study, we investigated 264 protein-coding sequences (CDSs) from four fertilization-related gene sets, (1) capacitation/decapacitation, (2) sperm motility, (3) sperm competition/fertilization, and (4) acrosomal reaction and zona reaction, in 49 representative taxa covering 20 major mammalian orders. We assessed the evolutionary trajectories of fertilization-related genes in scrotal and natural healthy "cryptorchid"

(UDT in the abdomen and IDT in the groin) mammals and predicted the functional alterations resulting from fixed amino acid mutations in cryptorchid species. Our results provide an in-depth theoretical understanding of cryptorchidism in humans and domestic animals, and more importantly, provide a basis for the development of new therapeutic targets and strategies (including gene therapy and stem cell therapy).

MATERIALS AND METHODS

Data Collection and Preparation

Capacitation, sperm motility, sperm competition, acrosomal reaction and zona reaction are the most vital processes during fertilization (Holt and Van Look, 2004; Doshi et al., 2012). Fertilization-related genes were obtained from the AmiGO database (Carbon et al., 2009)[1] by searches with the keywords (1) "capacitation" and "decapacitation," (2) "sperm motility," (3) "sperm fertilization" and "sperm competition," and (4) "acrosomal reaction" and "zona reaction." In total, 36, 101, 109, and 22 genes were obtained for each sperm function (**Supplementary Table 1**).

Further, CDSs of these genes in 49 mammalian species (including nine with UDT, 16 with IDT, and 24 with CDT) (**Supplementary Table 2**) were obtained from the *Senckenberg Genome Browser*[2] (Hecker and Hiller, 2020) using in-house Perl scripts. Among these 49 species, we chose (1) at least one representative species for each main mammalian order, (2) all the focused ascrotal (IDT + UDT) mammals, and (3) species with higher quality genomes if there were overrepresented CDT mammals in the same order. The longest transcript was chosen for downstream evolutionary analyses if more than one transcript was obtained for a single locus. Codon-based nucleotide sequences were aligned using MACSE with default parameters (Ranwez et al., 2011). Poorly aligned regions and gaps were trimmed using Gblocks setting "Gblocks −t = c-b4 = 5-b5 = h" (Talavera and Castresana, 2007). Finally, 35, 97, 108, and 22 alignments were generated (excluding *PAEP*, *HOATZ*, *SEPTIN12*, *CFAP251*, *DNAAF6*, and *NOX5* owing to alignment failures) (**Supplementary Table 1**).

Data on the testicular positions (i.e., CDT, IDT, and UDT) of representative mammals included in the study were obtained from the literature (Williams and Hutson, 1991; Hutson et al., 1992; Foresta et al., 2008; Kleisner et al., 2010; Lovegrove, 2014) and are provided in **Supplementary Table 2**. The working trees used for evolutionary and phylogenetic analyses were retrieved using TimeTree[3] (Kumar et al., 2017; **Supplementary Figure 1**).

Information on sperm motility, including VCL, VAP, VSL, LIN, ALH, STR, WOB, BCF, and total motility from CASA was collected from a literature search (**Supplementary Table 3**).

Comparison of Sperm Motility Among Mammals With Different Testicular Positions

To estimate differences in sperm motility among mammals with UDT, IDT, and CDT, ANOVA accounting for phylogenetic relationships was performed using the *phylANOVA* function in the phytools package in R (Revell, 2012), with a *p*-value cutoff of 0.05. The phylogenetic topology was obtained using TimeTree (see text footnote 3) (Kumar et al., 2017; **Supplementary Figure 1**).

Identification and Functional Prediction of Specific Fixed Amino Acid Substitutions in Ascrotal Mammals

FasParser (Sun, 2017) was used to identify fixed amino acid changes in natural ascrotal mammals (UDT + IDT, UDT, and IDT) compared with the sequences of scrotal counterparts. Further, to verify the specificity of the identified ascrotal mammal-specific fixed amino acid changes at a larger scale, alignments were expanded to include 106–117 mammals (49 mammals in the evolutionary analyses and additional taxa) (**Supplementary Table 4**).

Amino acid residues were classified by charge, polarity, and polarity plus volume as described previously in Zhang (2000). The impacts of fixed specific substitutions on function were predicted using PolyPhen-2[4] (Adzhubei et al., 2010), HOPE[5] (Venselaar et al., 2010), and SIFT[6] (Sim et al., 2012). PolyPhen-2 classifies amino acid substitutions as "benign," "possibly damaging," or "probably damaging" based on straightforward physical and comparative considerations. HOPE predicts the structural effects of a mutation. SIFT predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids (where a SIFT score of less than 0.05 indicates a deleterious effect).

Detection of Rapidly Evolving and Positively Selected Genes

The codeml tool in PAML (Yang, 2007) was employed to identify rapidly evolving genes (REGs) and positively selected genes (PSGs) in the lineages leading to UDT and IDT mammals.

First, a branch model was used to evaluate heterogeneity in evolutionary rates [non-synonymous-to-synonymous (d_N/d_S) ratio] between foreground branches (IDT and UDT lineages, separately) and background branches (the remaining lineages) (null model: model = 0; alternative model: model = 2). A gene with a significantly higher d_N/d_S ratio on foreground branches than on background branches was defined as a REG. Second, a branch-site model was used to test PSGs, with a null model in which the d_N/d_S ratio is fixed at 1 and an alternative model in which the d_N/d_S ratio is free to vary and positive selection is allowed (Yang, 1998) (null model: model = 2, NSsites = 2, fix_omega = 0; alternative model: model = 2, NSsites = 2,

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[1] http://amigo.geneontology.org/amigo
[2] https://genome.senckenberg.de//
[3] http://www.timetree.org/

[4] http://genetics.bwh.harvard.edu/pph2/
[5] https://www3.cmbi.umcn.nl/hope/
[6] https://sift.bii.a-star.edu.sg/

fix_omega = 1, omega = 1). Positively selected codons were identified by Bayes empirical Bayes with $p > 0.8$.

We corrected for multiple testing by using the Benjamini-Hochberg method for each gene within a set, with a cutoff of 0.05 (Benjamini and Hochberg, 1995).

To test whether the numbers of REGs in UDT and IDT mammals were greater than expected based on the number of fertilization-related genes in each set, gene overlap was evaluated using the *GeneOverlap* package in R (Shen, 2014). Fisher's exact test was used to evaluate independence based on a contingency table of gene counts in each set. The odds ratio was used to determine the strength of the association between two gene lists, where odds ratio >1 indicated a strong association and odds ratio <1 implied no association.

Association Between Molecular Evolution and Testicular Position by Phylogenetic Generalized Least Squares Regression

To assess the relationship between the rate of molecular evolution and testicular position, Phylogenetic Generalized Least Squares (PGLS) regression, which accounts for the phylogeny, was employed (Muntané et al., 2018). The root-to-tip d_N/d_S value was calculated for each gene and representative species and the association with the testicular position was evaluated based on a three-category classification (i.e., UDT, IDT, and CDT) and a two-category classification (ascrotal and scrotal testes).

Functional and Pathway Enrichment Analyses

Genes were evaluated by functional and signaling pathway enrichment analyses to explore the potential biological mechanisms underlying normal reproduction in ascrotal mammals. Pathways and Gene Ontology (GO) gene sets from Metascape[7] (Zhou et al., 2019) were used, taking *Homo sapiens* as both input and analysis species. GO enrichment was evaluated in the three general categories, molecular functions, biological processes, cellular components. Pathway enrichment was evaluated based on databases (including GO biological processes, reactome gene sets, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, WikiPathways, canonical pathways, and hallmark gene sets) built in Metascape.

RESULTS

Sperm Motility Traits Are Similar in Ascrotal and Scrotal Mammals

We compared sperm motility parameters between ascrotal (UDT and IDT groups) and scrotal mammals. Of the nine parameters (i.e., VCL, VAP, VSL, LIN, ALH, STR, WOB, BCF, and total motility), eight parameters (VCL, VAP, VSL, LIN, ALH, WOB, BCF, and total motility) did not differ significantly between UDT, IDT and CDT mammals. STR was significantly higher in UDT

[7]http://metascape.org

and CDT mammals than in IDT counterparts (adjusted $p = 0.030$ and 0.015, respectively) (**Figure 1**).

Fixed Amino Acid Substitutions in Fertilization-Related Proteins of Undescended Testes Mammals Affect Protein Properties

None of the genes associated with fertilization evaluated in the present study showed fixed amino acid changes specific to all ascrotal (UDT + IDT) mammals. We identified three UDT mammal-specific substitutions in three proteins (**Figure 2**): CFAP65 V1599S, CFAP157 A34D, and FSIP2 D785H. These three substitutions resulted in radical changes in protein properties; CFAP65 V1599S changed a non-polar valine to a polar serine, a neutral and non-polar alanine was replaced with a negative and polar aspartic acid in CFAP157 A34D, and a negative and relatively small aspartic acid was replaced with a positive and relatively large histidine in FSIP2 D785H. Using an expanded dataset including 106–117 mammals, the specificity of the three fixed substitutions in UDT mammals was generally supported, with some exceptions (**Supplementary Figure 2**).
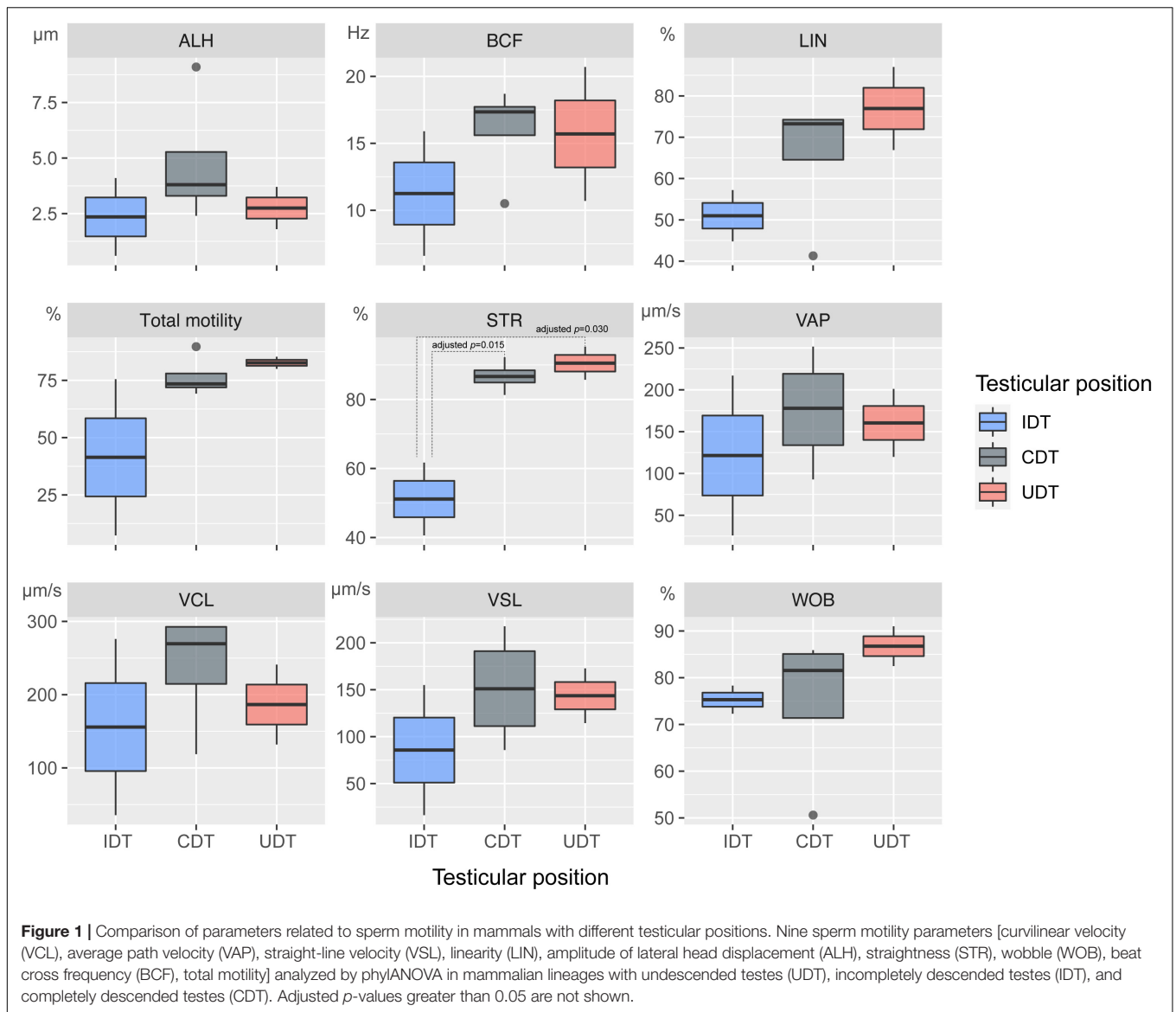
In silico analyses showed that some of the UDT mammal-specific changes were predicted to alter protein functions as well as additional physicochemical properties (**Figure 3**). For example, all three amino acid changes could affect the volume of proteins; the mutant serine in CFAP65 V1599S and aspartic acid in CFAP157 A34D would make proteins less hydrophobic and affect the conformation or secondary structure of proteins.

Rapidly Evolving Genes and Positively Selected Genes in Ascrotal Mammals Contribute to Sperm Motility and Competition or Fertilization

Two genes in the capacitation set, five genes in the sperm motility set, nine genes in the sperm competition/fertilization set, and four genes in the acrosomal reaction/zona reaction set were identified as REGs in UDT mammals. For comparison, three genes were associated with capacitation, 11 genes were associated with sperm motility, 11 genes were associated with sperm competition/fertilization, and two were associated with acrosomal reaction/zona reaction in IDT mammals (**Supplementary Table 5**). Additionally, we used a gene overlap analysis to assess whether the number of REGs was greater than expected based on the numbers in each set of fertilization-related genes. The number of REGs identified in each gene set was significantly greater than expected by chance (**Supplementary Figure 3**); most REGs were associated with sperm motility and competition/fertilization. Moreover, of these REGs, *DNAH11*, and *FSIP2* related to sperm motility and *SPAM1* related to sperm competition/fertilization overlapped in UDT and IDT mammals (**Figure 4A**).

As for PSGs, in UDT mammals, we detected the signature of positive selection in 5, 8, and 13 genes in the capacitation, sperm motility, and sperm competition/fertilization sets, respectively. By contrast, only two genes in the sperm motility set and six genes

Figure 1 | Comparison of parameters related to sperm motility in mammals with different testicular positions. Nine sperm motility parameters [curvilinear velocity (VCL), average path velocity (VAP), straight-line velocity (VSL), linearity (LIN), amplitude of lateral head displacement (ALH), straightness (STR), wobble (WOB), beat cross frequency (BCF), total motility] analyzed by phylANOVA in mammalian lineages with undescended testes (UDT), incompletely descended testes (IDT), and completely descended testes (CDT). Adjusted p-values greater than 0.05 are not shown.

in the sperm competition/fertilization set underwent positive selection in IDT mammals (**Supplementary Table 5**). *ADAM32* and *GLIPR1L2* related to sperm competition/fertilization were shared REGs in UDT and IDT mammals (**Figure 4B**).

Combining results for REGs and PSGs, 31 genes were subjected to adaptive evolution in UDT mammals and 25 genes underwent adaptive evolution in IDT mammals (**Supplementary Table 5**). Enrichment analyses revealed that although several enriched terms and pathways were shared between UDT and IDT groups (e.g., single fertilization, acrosomal vesicle, and cellular process involved in reproduction in multicellular organism), the 31 and 25 genes experiencing adaptive evolution were enriched in clusters of distinct terms and pathways. In particular, enriched GO categories for the 31 genes in UDT mammals included acrosome reaction, fusion of sperm to egg plasma membrane and epithelial cilium movement involved in extracellular fluid movement; the 25

genes under adaptive evolution in IDT were additionally enriched for sperm midpiece, hexosaminidase activity, and regulation of cilium movement (**Figure 5** and **Supplementary Tables 6, 7**).

Fertilization-Related Gene Evolution Was Associated With Mammalian Testicular Position

By PGLS regression, we identified 22 and 9 genes associated with the three-category and two-category classifications of mammalian testicular position, respectively (**Table 1**). Of these genes, the evolution of *ACRBP* and *NPHP4* related to sperm motility and *LY6K* and *SORD* related to sperm competition/fertilization was associated with mammalian testicular position based on both the three-category and two-category classification.
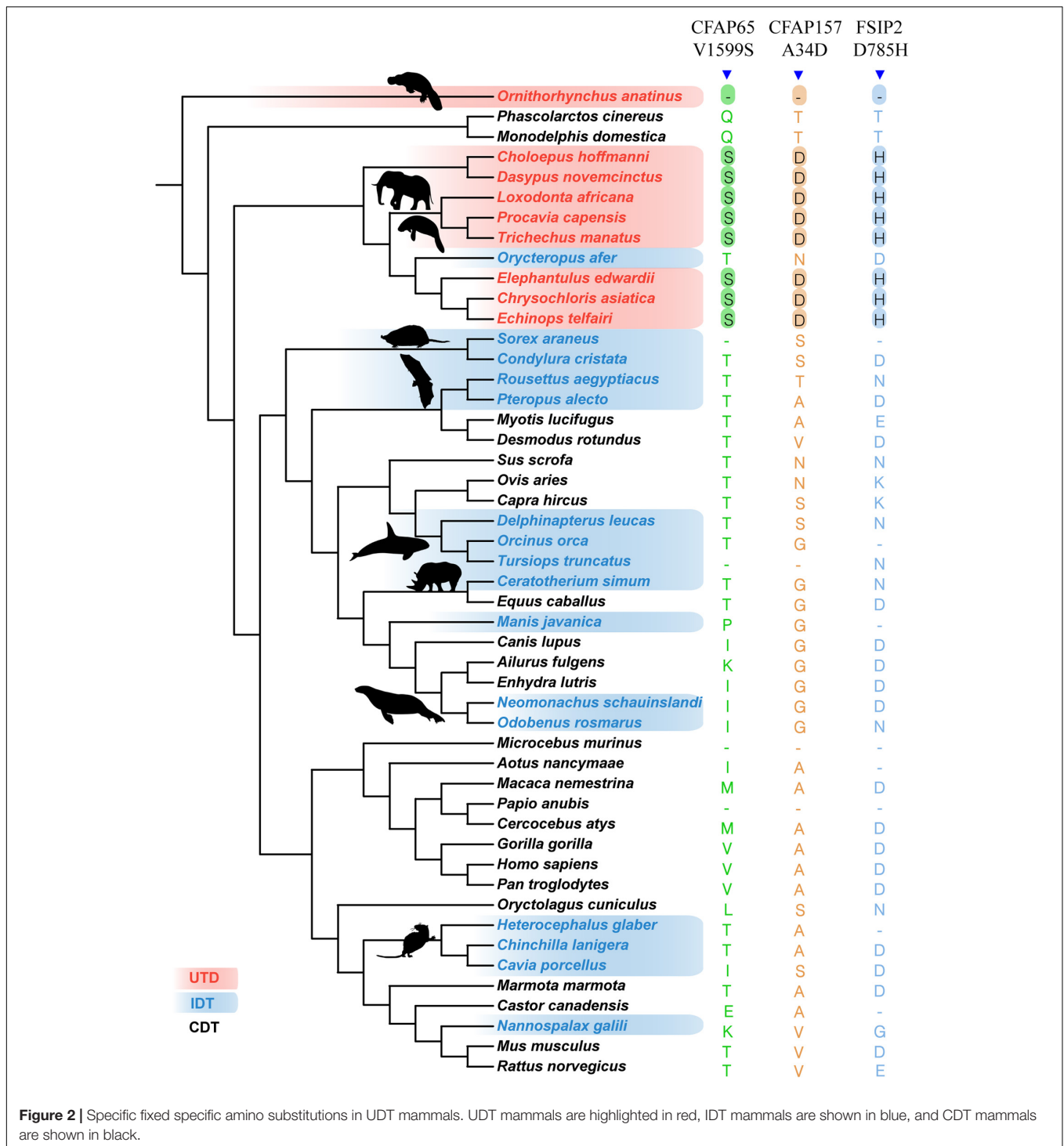
Figure 2 | Specific fixed specific amino substitutions in UDT mammals. UDT mammals are highlighted in red, IDT mammals are shown in blue, and CDT mammals are shown in black.
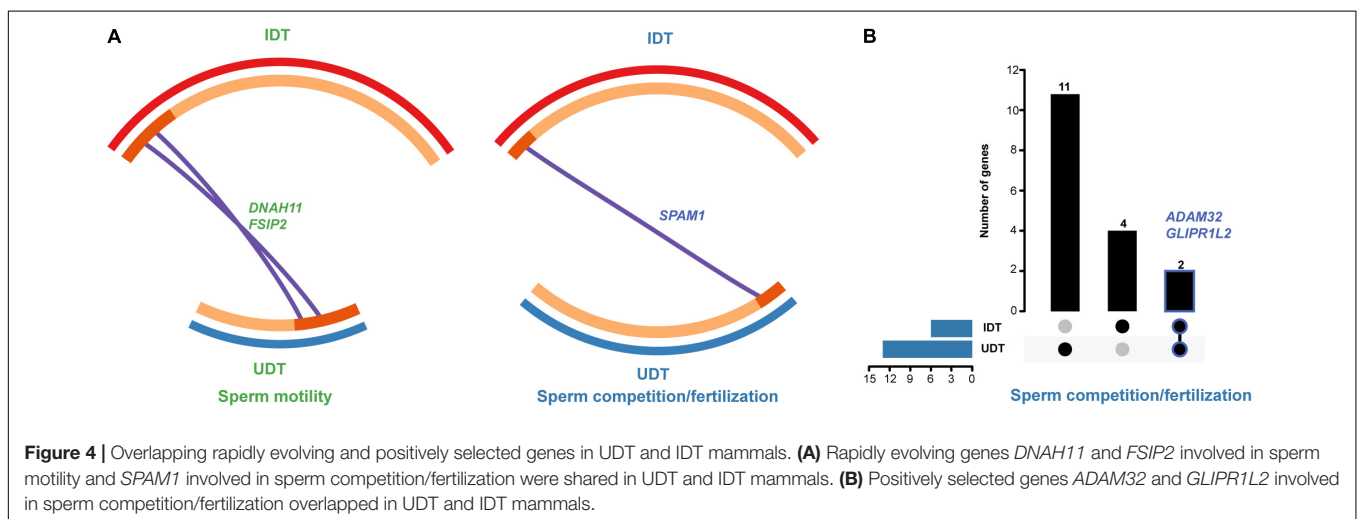
DISCUSSION

Comparable Sperm Motility in Ascrotal and Scrotal Mammals

Humans and domestic animals with cryptorchidism (or undescended testes) have an elevated risk of infertility. The incidences of azoospermia are 13 and 89% in unilateral and

untreated bilateral cryptorchidism, respectively (Hadziselimovic and Herzog, 2001). Although early hormonal and surgical interventions can be performed at appropriate time points, the overall success rate varies and individuals remain at elevated risks of male sterility and testicular cancer (Chung and Brock, 2011). It has been reported that ~10% of patients with infertility have a history of cryptorchidism and orchidopexy (Grasso et al., 1991).
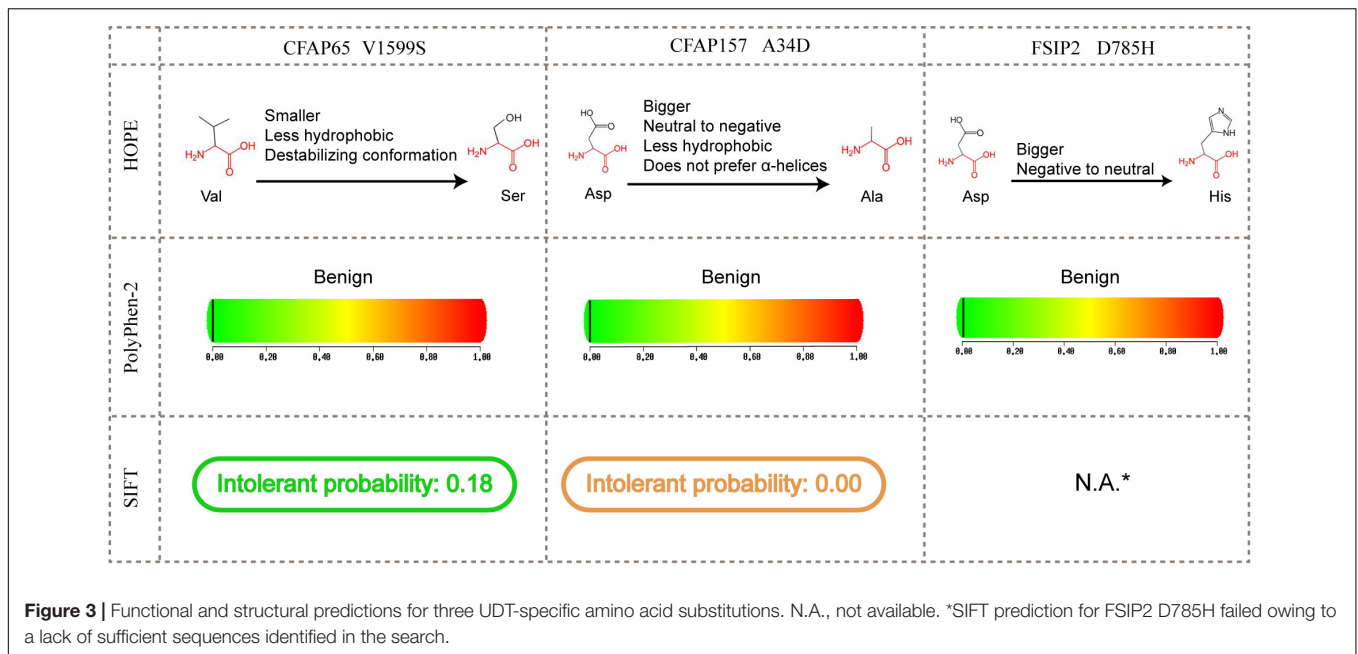
Figure 3 | Functional and structural predictions for three UDT-specific amino acid substitutions. N.A., not available. *SIFT prediction for FSIP2 D785H failed owing to a lack of sufficient sequences identified in the search.

Figure 4 | Overlapping rapidly evolving and positively selected genes in UDT and IDT mammals. **(A)** Rapidly evolving genes *DNAH11* and *FSIP2* involved in sperm motility and *SPAM1* involved in sperm competition/fertilization were shared in UDT and IDT mammals. **(B)** Positively selected genes *ADAM32* and *GLIPR1L2* involved in sperm competition/fertilization overlapped in UDT and IDT mammals.
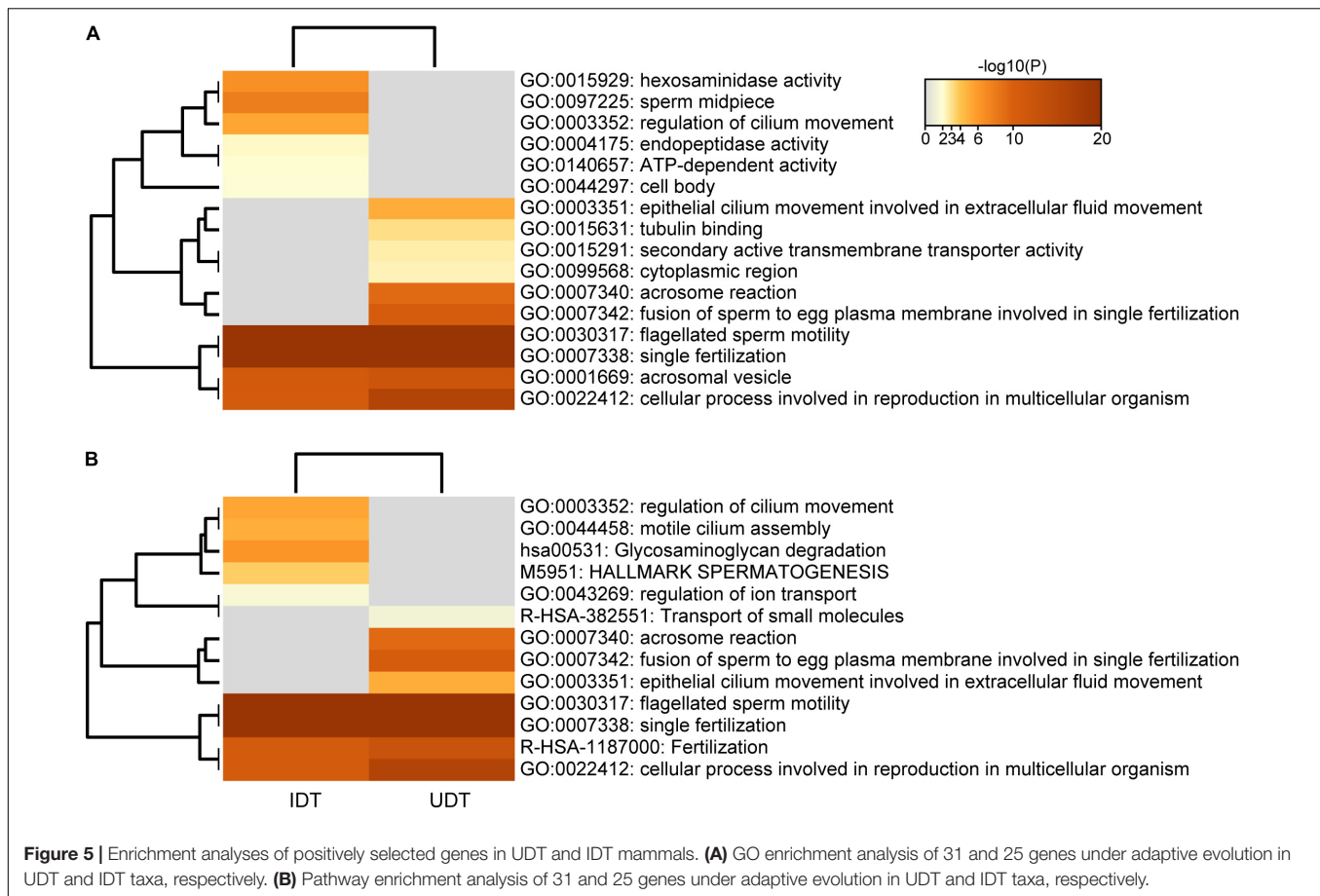
However, reports on the decreased motility of sperm or infertility in natural ascrotal mammals are rare relative to studies of scrotal mammals. In the present study, to estimate sperm motility in UDT, IDT, and CDT mammals, we first compared various parameters related to sperm motility. From a data perspective, we confirmed that sperm mobility was not significantly different between ascrotal and scrotal mammals, as reported in previous studies (**Figure 1**).

Molecular Evolution of Sperm Motility- and Competition-Related Genes Benefits to Male Fertility in Natural "Cryptorchid" Mammals

A series of evolutionary analyses of fertilization-related genes revealed distinct evolutionary trajectories in scrotal and ascrotal groups, providing insight into the molecular mechanisms underlying normal sperm motility and fertility in natural "cryptorchid" mammals. We found that genes related to fertilization showed ascrotal mammal-specific evolutionary signals (including rapid evolution and positive selection, fixed amino acid substitutions, and significant associations with testicular position) (**Figures 2**, **4**, **Table 1**, and **Supplementary Table 5**), implying the importance of all general functions (i.e., capacitation, sperm motility, sperm competition, and acrosomal reaction/zona reaction) in fertilization in ascrotal mammals. Genes with ascrotal mammal-specific evolutionary signals were disproportionately related to sperm motility and sperm competition/fertilization, suggesting that these two sperm functions underwent stronger selection to recover normal reproductive functions under the "cryptorchid" condition.

Figure 5 | Enrichment analyses of positively selected genes in UDT and IDT mammals. **(A)** GO enrichment analysis of 31 and 25 genes under adaptive evolution in UDT and IDT taxa, respectively. **(B)** Pathway enrichment analysis of 31 and 25 genes under adaptive evolution in UDT and IDT taxa, respectively.

We found that the sperm motility-related genes *DNAH11* and *FSIP2* and sperm competition/fertilization-related genes *SPAM1*, *ADAM32*, and *GLIPR1L2* underwent adaptive evolution in both the UDT and IDT lineages (**Figure 4**), providing evidence for convergent evolution as well as the necessity of these genes in natural "cryptorchid" mammals. A Disintegrin And Metalloproteinase Domain 32 (*ADAM32*) is a member of the ADAM family, expressed predominantly in the testis (Choi et al., 2003). ADAM32 has a potential role in sperm fertilization, with evidence for positive selection across mammals (Civetta, 2003; Glassey and Civetta, 2004). In the present study, amino acid residue 397 of ADAM32 was identified as a positively selected site in both UDT and IDT mammals (**Supplementary Table 5**). Importantly, this site is located in a disintegrin domain with a known egg integrin ligand involved in sperm-egg adhesion leading to fusion (Yuan et al., 1997). Positive selection on ADAM32 397 within the disintegrin domain in all ascrotal mammals might increase sperm fertilization via sperm-egg fusion.

Fibrous Sheath Interacting Protein 2 (*FSIP2*) is a rapidly evolving gene in ascrotal mammals and harbored a UDT mammal-specific amino acid substitution (D785H) (**Figure 2**). The protein encoded by *FSIP2* is associated with the formation of the sperm fibrous sheath. Patients with mutant FSIP2 have been diagnosed with multiple morphological abnormalities of

the sperm flagella (MMAF), causing sperm motility disorders and male infertility (Liu et al., 2021). Although FSIP2 D785H is not within any known functional domains, our results suggest that the mutation has a functional effect in ascrotal mammals. Further functional assays and studies of the mechanism by which FSIP2 D785H contributes to the regulation of sperm motility and reproduction in ascrotal mammals are needed.

Two fixed specific amino acid substitutions (CFAP65 V1599S and CFAP157 A34D) in UDT mammals were involved in sperm motility (**Figure 2**). *CFAP65* and *CFAP157* are members of the cilia and flagella associated protein family, and both are specifically required during spermatogenesis for flagellum morphogenesis and sperm motility. In humans and *Cfap65*-mutated mice, biallelic mutations in CFAP65 (p.Arg1619* and p.Leu1757*) cause MMAF and impair sperm motility (Li et al., 2020). The UDT-specific amino acid change CFAP65 V1599S identified in the present study was near the known mutations linked to sperm motility, indicating a potential contribution to normal sperm motility in ascrotal mammals.

In humans, initial testicular position is considered a risk factor for infertility in individuals with a history of cryptorchidism (Simoni and Huhtaniemi, 2017). It has been suggested that testes that are initially located beyond the inguinal canal are favorable for fertility. Patients with cryptorchidism with palpable testes (i.e., testes placed in the superficial inguinal pouch) have a

TABLE 1 | Fertilization-related genes associated with mammalian testicular position.

Testicular position classification*	Sperm function**	Gene	Lambda	Coefficient	r^2	*p*-value
Three-category	(3)	KCNU1	1	0.93647	0.26310	0.00012
Three-category	(2)	CFAP157	1	2.61071	0.17420	0.00167
Three-category	(3)	LY6K	1	2.37623	0.16720	0.00207
Three-category	(2, 3)	APOB	1	1.29652	0.16040	0.00254
Three-category	(3)	SORD	1	2.48876	0.10470	0.01334
Three-category	(2)	ENKUR	1	1.43330	0.09880	0.01587
Three-category	(2)	ACRBP	1	2.95155	0.09526	0.01761
Three-category	(3, 4)	AKAP3	1	1.23231	0.09383	0.01836
Three-category	(2)	NPHP4	1	1.84430	0.08897	0.02234
Three-category	(2, 3)	SMCP	1	2.11964	0.08404	0.02446
Three-category	(2)	INPP5B	1	1.61370	0.07873	0.02858
Three-category	(3)	FOLR2	1	2.45664	0.07401	0.03282
Three-category	(2)	LRRC6	1	3.41978	0.07254	0.03427
Three-category	(2)	TACR2	1	2.64913	0.07190	0.03491
Three-category	(1)	PEBP1	1	1.18871	0.06405	0.04398
Three-category	(2)	TAC4	1	2.35563	0.06396	0.04409
Three-category	(1)	SPA17	1	1.62510	0.06104	0.04805
Three-category	(1, 2)	INTS13	1	2.37408	0.06093	0.04820
Two-category	(3)	LY6K	1	1.81224	0.14100	0.00455
Two-category	(1, 3)	ELSPBP1	1	1.65716	0.10600	0.01283
Two-category	(2)	ACRBP	1	2.30480	0.08743	0.02215
Two-category	(3)	SORD	1	1.89300	0.08615	0.02299
Two-category	(1, 2)	EFCAB9	1	0.89938	0.08233	0.02571
Two-category	(2)	CELF3	1	2.18172	0.06808	0.03907
Two-category	(2)	NPHP4	1	1.54985	0.06236	0.04804

*Three-category classification of the testicular position: UDT, IDT, and CDT; two-category classification of the testicular position: ascrotal and scrotal testes.
**(1) capacitation and decapacitation; (2) sperm motility; (3) sperm fertilization and sperm competition; (4) acrosomal reaction and zona reaction.

higher maximal sperm density, a large testicular size, and higher paternity rate than those of intracanalicular or abdominal cases (Puri and Sparnon, 1990; Cortes and Thorup, 1991; Mayr et al., 1996).

In analogy with different degrees of testicular descent in cryptorchidism, different testicular positions between natural IDT and UDT mammals might challenge reproductive health by varying degrees. Evolutionarily, natural UDT mammals likely faced many serious challenges from a physiological perspective due to the undescended testis (high cryptorchidism). Therefore, more fertilization-related genes may undergo adaptive evolution and accumulated modifications. We observed more genes exhibiting the signature of adaptive evolution (REGs and PSGs) in UDT mammals than in IDT mammals (31 and 25 genes, respectively). These two sets of genes were enriched for different clusters of functional terms and signaling pathways (**Supplementary Tables 5–7**). Although these genes collectively contributed to final fertility, the evolutionary patterns of individual fertilization-related genes differed among mammals with various testicular positions.

with different testicular positions. Additionally, we detected heterogeneous evolutionary patterns of hundreds of fertilization-related genes between natural ascrotal and scrotal mammals. Combined with lineage-specific fixation and positive selection on certain amino acids in ascrotal mammals, our results suggested that genes and processes associated with sperm motility and sperm competition/fertility in natural "cryptorchid" mammals, especially in UDT species, experienced more frequent and stronger selective pressure. These observations suggest that UDT mammals have a more challenging physiological testicular environment and unique genetic solutions. These findings provide insight into the molecular mechanisms underlying normal sperm motility and fertilization in natural ascrotal mammals and improve our general understanding of the pathology of cryptorchidism from a molecular evolutionary perspective. Finally, our results may be helpful in establishing a genetic diagnosis for dysfunctions in fertility and sperm motility due to congenital cryptorchidism and provide candidate target loci for genetic counseling and infertility treatment in humans and other domestic animals.

CONCLUSION

In summary, we provide the first demonstration that sperm motility does not differ significantly among mammals

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and

accession number(s) can be found below: https://figshare.com/ and https://doi.org/10.6084/m9.figshare.17059700.v1.

AUTHOR CONTRIBUTIONS

SX, GY, and WR conceived the study and assisted with manuscript revision. SC and RT participated in the data analysis. SC interpreted the data and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021. 828325/full#supplementary-material

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