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RESEARCH

Comparative Genomics Analysis Provides Insights into Pregnancy Maintenance Mechanisms in the Ovoviviparous Teleost Black Rockfish (*Sebastes schlegelii*)

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Abstract

The black rockfish (*Sebastes schlegelii*), an economically important ovoviviparous teleost in Chinese aquaculture, exhibits a distinctive reproductive strategy characterized by internal fertilization and prolonged embryonic development within females until live birth. However, this reproductive mode poses substantial challenges in artificial breeding practices due to a poor understanding of pregnancy (hereafter referring to intraovarian gestation in *S. schlegelii*) maintenance mechanisms. Here, we conducted a comparative genomic analysis between *S. schlegelii* and representative oviparous and ovoviviparous fishes, identifying 374 lineage-specific gene families associated with cellular signaling, metabolic regulation, immune response, and developmental processes. In addition, 73 gene families with significant expansions were identified to be mainly involved in extracellular matrix (ECM) structure and regulation, and neuromodulation. Furthermore, 164 genes potentially associated with mitochondrial function, metabolic pathways and angiogenesis were subjected to strong positive selection. Notably, expression profiles of candidate functional genes associated with ECM structure and regulation, and angiogenesis were detected using transcriptomic data of stage V ovary, optic vesicle stage, and pigmentation stage for ovarian stroma tissue, highlighting the vital roles of ECM remodeling and angiogenesis during pregnancy. These findings provide novel insights into the molecular mechanisms underlying pregnancy maintenance in *S. schlegelii* and contribute to a broader understanding of the genetic adaptations with pregnancy in ovoviviparous teleosts.

Keywords *Sebastes schlegelii* · Comparative genomics · Pregnancy maintenance · Gene expression

Introduction

As a vital process in the life history of organisms, reproduction has also been a central life-history trait driven by adaptive evolution. To adapt to diverse ecological pressures, vertebrates have developed various reproductive strategies, such as oviparity, ovoviviparity, and viviparity (Giesel 1976). Among these, viviparity has evolved independently multiple times, especially in aquatic and extreme environments (Blackbum 1999), and ovoviviparity is often regarded as an intermediate stages towards true viviparity (Angelini & Ghiara 1984). Fishes, as an ancient and pivotal vertebrate lineage, exemplify this evolutionary diversity. For instance, chondrichthyans universally utilize internal fertilization, displaying reproductive modes from egg-laying shortly after fertilization to prolonged egg retention culminating in live birth (Wourms 1977). In contrast, most teleosts predominantly employ external fertilization, requiring synchronized gamete release into the aquatic environment (Blackbum 1999; Patzner 2008). Nonetheless, internal fertilization has independently arisen in specific teleost lineages possessing specialized copulatory organs for sperm transfer (Wootton & Smith 2014). Fertilized eggs in these teleosts typically develop internally within the ovary until parturition, though some species, such as *Compsura heterura*, the eggs are released shortly after fertilization (Fukakusa et al. 2020; Smith & Wootton 2016).

Ovoviviparity represents an evolutionarily intermediate reproductive strategy characterized by internal retention and development of fertilized eggs until parturition of

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free-swimming larvae (Wourms 1981). Observed widely among fishes, reptiles, and amphibians (Halliwell et al. 2017; Shine & Bull 1979; Wen 2020), ovoviviparity typically involves low egg nutrient storage and incipient placental structures resembling those in viviparous species (Wourms 1981; Wourms et al. 1988). This strategy provides adaptive advantages by protecting embryos from environmental stressors and predation, while maintaining maternal nutrient provisioning (Blackbum 1999). However, these benefits come with trade-offs, including higher maternal metabolic costs, offspring loss from maternal mortality, and constrained fecundity (Wourms & Lombardi 1992). Ovoviviparous fish reproduction encompasses three main phases: copulation, gestation, and parturition. Male maturity typically precedes female maturity, and sperm is transferred during mating and stored within the female reproductive tract until environmental cues trigger fertilization and embryonic development (Koya et al. 2003; Mori et al. 2003).

The black rockfish (*Sebastes schlegelii*), belonging to the family Sebastidae of the order Scorpaeniformes (Betancur-R et al. 2017), is an economically important marine aquaculture species in China, Korea and Japan (Chen et al. 2021). As an ovoviviparous teleost, it exhibits internal fertilization and prolonged embryonic development within females until live birth. Although this reproductive strategy may represent an adaptive advantage in evolutionary terms, it poses considerable challenges for artificial breeding, as the entire reproductive process depends critically on the ovarian microenvironment. Disturbances in this environment can lead to fertilization failure, reduced larval yield, developmental anomalies causing stillbirths, and dysregulated parturition resulting in dystocia or pregnancy termination. These issues severely impact reproductive efficiency, germplasm conservation, and genetic improvement, highlighting the importance of elucidating the molecular mechanisms underlying intraovarian gestation in *S. schlegelii*. Previous studies have investigated the physiological requirements and molecular mechanisms of mating (Lyu et al. 2022, 2024a; Yao, et al. 2023), pregnancy maintenance (Guo et al. 2024; Zheng et al. 2023), and parturition (Lyu et al. 2024b; X. Wang et al. 2022; Xie et al. 2023; Yan et al. 2022, 2023). However, these investigations have mainly targeted isolated genes or pathways, leaving comprehensive multi-omics analyses of pregnancy maintenance in this species.

Therefore, in this study, a comparative genomic analysis was established between *S. schlegelii* and other representative oviparous (e.g., *Danio rerio*, *Oryzias latipes*) and ovoviviparous (e.g., *Poecilia latipinna*, *Gambusia affinis*) fish species to identify lineage-specific gene families, expanded gene families, and positively selected genes relevant to its reproductive adaptations. Furthermore, transcriptomic profiling of ovarian stromal tissues across reproductive stages in *S. schlegelii* was performed to generate candidate molecular pathways, thereby providing a foundation for understanding the mechanisms of intraovarian gestation in this species.

Materials and Methods

Phylogenetic Analyses and Divergence Time Estimation

To understand phylogenetic relationship and divergence time of *S. schlegelii* with other fish, coding sequence (CDS) predicted from genome assemblies of nine representative fish species were downloaded from NCBI, including six ovoviviparous taxa: *S. schlegelii* (Scorpaenidae), four poeciliids (*P. latipinna*, *P. reticulata*, *G. affinis*, and *Xiphophorus maculatus*), *Rhincodon typus* (Rhincodontidae), and three oviparous taxa: *D. rerio* (Cyprinidae), *O. latipes* (Adrianichthyidae), and *Oncorhynchus mykiss* (Salmonidae). All genome assemblies and corresponding accession numbers were retrieved from the NCBI Genome database in September 2023, and detailed information is provided in Supplementary Table 8. The longest CDS per gene was extracted using an in-house Python script and subsequently translated into protein sequences. Gene families were then clustered into orthologous groups across the nine selected fish species using OrthoFinder (v2.4.0) with its built-in Diamond algorithm (Emms & Kelly 2015). In addition, the sequences of conserved single-copy orthologous genes shared by nine fish species were selected for multiple sequence alignment using MUSCLE (v5.1) (Edgar 2022), and Gblocks was used to extract conserved sequences and filter unconserved sites (Talavera & Castresana 2007). Furthermore, IQ-TREE (v2.0.3) was used to infer phylogenetic relationships based on a concatenated multiple sequence alignment using maximum likelihood (ML) method (Minh et al. 2020). The optimal substitution model was selected via ModelFinder Plus (MFP) (Kalyaanamoorthy et al. 2017) with Bayesian Information Criterion (BIC) as the model selection criterion. The ML phylogenetic tree was rooted with the cartilaginous fish *R. typus* designated as the outgroup, and visualized using the Interactive Tree of Life (iTOL) platform (v6.7.4) (Letunic & Bork 2021). Divergence times were estimated using the MCMCTREE algorithm implemented in PAML (v4.9) (Yang 2007) using phylogenetic tree and alignment results, and a time calibration was performed using fossil constraints obtained from the TimeTree database (Kumar et al. 2017).

Expansion and Contraction Analyses of Gene Families

To understand the contributions of gene gains and losses for functional changes, gene families that expanded or contracted among the nine selected species were analyzed using CAFE (v5.0) according to the orthologs and phylogenetic tree (Mendes et al. 2021). Gene families containing over 100 copies in any single species were excluded from the analysis, as such unusually large families often represent repetitive elements, rapidly evolving receptor families, or annotation artifacts that may bias the inference of expansion/contraction in CAFE. Simulated random rates of birth and death were then used to predict gene family variations across phylogenetic tree lineage, with a significance threshold of a p-value < 0.05. Subsequently, to investigate the functional roles of genes within these families to pregnancy maintenance mechanisms for *S. schlegelii*, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed on expanded and contracted gene families for *S. schlegelii* using KOBAS (v3.0) (Bu et al. 2021). *Homo sapiens* was selected as the reference organism due to its well-annotated genome and comprehensive coverage of pregnancy-related pathways. The top 20 enriched GO and KEGG terms were visualized using ggplot2 package.

Selected Pressure Analysis

To identify candidate genes under the influence of positive selection for *S. schlegelii*, multiple sequence alignment was analyzed to detect selection signals on single-copy orthologs using MUSCLE (v5.1). The branch-site model in the CODEML program of PAML (v4.9) was implemented to conduct positive selection analysis (Yang 2007). This approach compares two evolutionary models: a null model restricting ω (the ratio of non-synonymous [dN] to synonymous [dS] substitutions, also known as Ka/Ks) ≤ 1 and an alternative model allowing $\omega > 1$ on specified branches. Likelihood-ratio tests (LRTs) were conducted on each model pair to determine significant positive selection sites in genes with the following criteria: (1) statistically significant LRT results ($P < 0.05$ after Bonferroni correction), and (2) branch-specific $\omega > 1$ in the alternative model. Evolutionary regimes were classified as: positive selection ($\omega > 1$), neutral evolution ($\omega = 1$), or purifying selection ($\omega < 1$). In the branch-site model, the ovoviviparous lineage *S. schlegelii* was specified as the foreground branch for positive selection analysis, while all other lineages were treated as the background. Functional enrichment analysis of positively selected genes (PSGs) was subsequently conducted using KOBAS (v3.0) with Fisher's exact test, retaining significantly enriched GO terms and KEGG pathways at a false discovery rate (FDR) < 0.05.

Candidate Functional Gene Expression Pattern Analysis

Ovarian stroma tissues of *S. schlegelii* were collected from three independent females at each of three developmental stages (stage V ovary, optic vesicle stage, and pigmentation stage), resulting in nine biological samples in total (3 stages × 3 biological replicates). Detailed developmental stage definitions and representative histological images are provided in the Supplementary Figure. Total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity and concentration were assessed with a Biodropsis BD-1000 nucleic acid analyzer (OSTC, Beijing, China) and gel electrophoresis. Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, first-strand cDNA was synthesized with random hexamer primers, followed by second-strand cDNA synthesis. The cDNA libraries were then constructed through end repair, A-tailing, adapter ligation, size selection, PCR amplification, and purification. After quality control, sequenced as 150 bp paired-end reads on the Illumina NovaSeq™ 6000 platform. The raw reads were filtered using Trimmomatic (v0.39) software to remove adapters and low-quality sequences. Subsequently, high-quality clean reads were mapped to the reference genome of *S. schlegelii* in NCBI (PRJNA516036) using Hisat2 (v2.2.1) software and the mapped reads from alignments were sorted using samtools (v1.6) software. The gene expression levels were quantified and normalized to fragments per kilobase of transcript per million fragments mapped (FPKM) by StringTie (v2.1.7) software. Finally, TBtools (v1.082) software was used to display heatmaps of gene expression levels using $\log_2$(FPKM). For Fig.5a and Fig.5b, mean expression values across three biological replicates per stage were used for visualization, whereas replicate-level expression values are reported in Supplementary Tables 6 and 7, respectively.

Results

The Identification of Orthologous Genes and Specific Gene Families for *S. schlegelii*

Gene families clustering for *S. schlegelii*, *P. latipinna*, *P. reticulata*, *G. affinis*, *X. maculatus*, *R. typus*, *D. rerio*, *O. latipes*, and *O. mykiss* were first conducted to identify orthologous genes. A total of 20,984 orthologous gene families

across the analyzed species were identified, with 10,794 core gene families conserved in all nine taxa (Table S1). In addition, comparative genomic analysis of *S. schlegelii*, with *O. latipes*, *D. rerio*, *P. reticulata*, *R. typus* revealed 374 candidate lineage-specific gene families in *S. schlegelii* (Fig. 1). Functional annotation through integrated GO terms and KEGG pathway enrichment analyses demonstrated that these unique gene families are predominantly enriched in cellular signaling, metabolic regulation, immune response, and developmental processes (Table S2). Specifically, GO term analysis highlighted the involvement of these gene families in immune-related processes (e.g., regulation of inflammatory responses, neutrophil migration, and T cell activation), and several cellular functions (e.g., calcium ion transport, cell adhesion, and the regulation of cytokine production) (Table S2). KEGG analysis identified significant enrichment in neural synaptic transmission, hormone signaling pathways, and immune system networks (Table S2).

Phylogenetic Relationship Construction and Divergence Time Estimate

To examine the evolutionary relationships of *S. schlegelii* and other fish species, 2,242 strict single copy orthologs from 10,794 core gene families were selected for phylogenetic construction and divergence times estimation (Fig. 2a). The resulting phylogeny recovered the expected separation of cartilaginous fish (*R. typus*) from ray-finned fishes, followed by the divergence of cyprinids (*D. rerio*) and salmonids (*O. mykiss*). *S. schlegelii*, from the Scorpaenidae lineage, diverged from *O. mykiss* approximately 231 million years ago (MYA), consistent with previously reported ranges (176–264 MYA) (Li et al. 2018; Rabosky et al. 2018). The poeciliid fishes (*P. reticulata, P. latipinna, G. affinis, X. maculatus*) formed a distinct clade, which split from *O. latipes* at ~ 132 MYA. Overall, the topology is congruent with established teleost phylogeny. Supporting the reliability of our divergence time estimates. Notably, *S. schlegelii* exhibited the closest evolutionary affinity to oviparous taxa among the examined ovoviviparous lineages, suggesting unique evolutionary trajectories of viviparity in this species.

Expansion and Contraction of Gene Families for *S. schlegelii* Genome

To investigate the genomic adaptations underlying pregnancy maintenance mechanisms in *S. schlegelii*, expansion and contraction analyses of gene families were performed among the above nine species using CAFE (v5.0). Our results revealed that 722 and 1295 gene families were expanded and contracted, respectively, in *S. schlegelii* (Fig. 2b). Among them, 73 gene families exhibited significant expansion and 347 showed significant contraction ($p < 0.05$) (Table S3). Enrichment analyses revealed that the 73 significantly expanded gene families (comprising 276 genes) (Table S4) were associated with a broad range of biological functions. Of which, several critical biological processes including ECM structure and regulation (e.g., ECM structural constituent, collagen-activated tyrosine kinase receptor signaling pathway, ECM organization), neuromodulation (e.g., G protein-coupled glutamate receptor signaling pathway, regulation of synaptic transmission, glutamatergic and peripheral nervous system myelin maintenance), transmembrane transport (e.g., ligand-gated ion

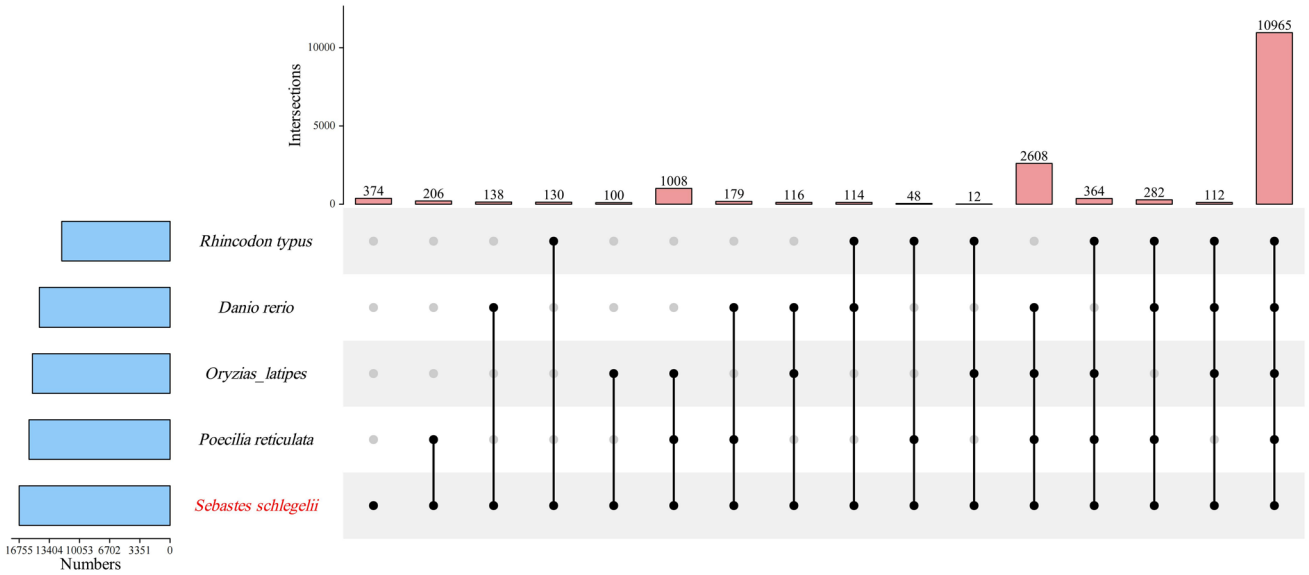

Fig. 1 UpSetPlot of gene orthologues shared among *S. schlegelii*, *O. latipes*, *D. rerio*, *P. reticulata*, and *R. typus*

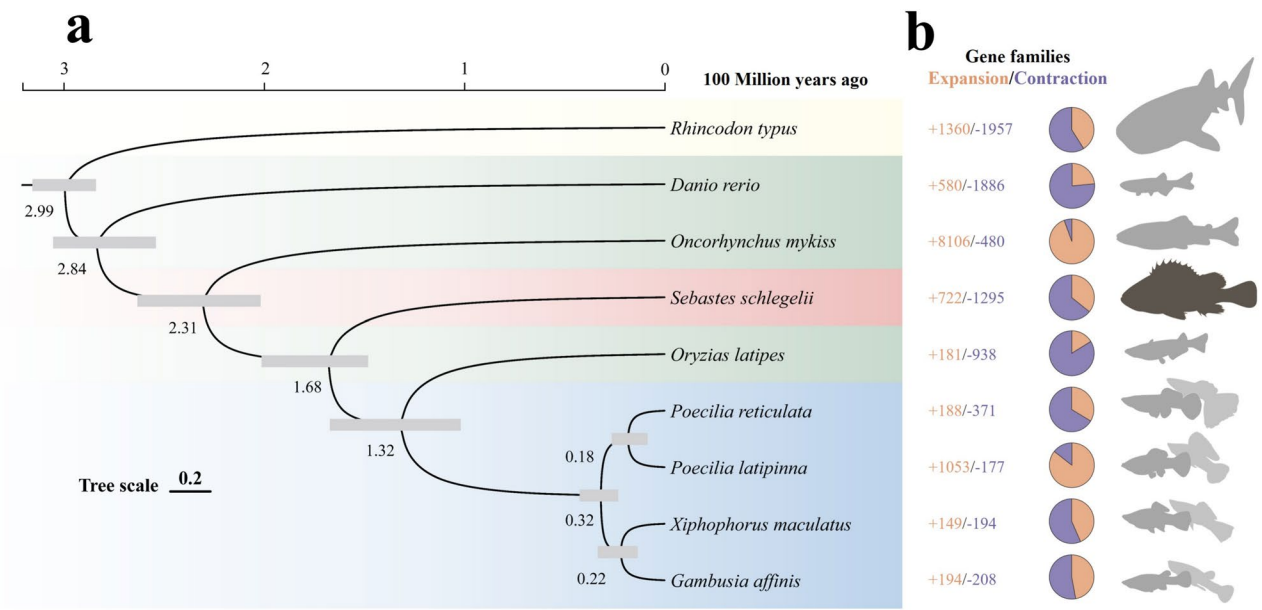

Fig. 2 **(a)** Phylogenetic relationship and divergence time of the nine fish species lineages, **(b)** The number of expanded (yellow) and contracted (purple) gene families of the nine fish species

channel activity) and membrane integration systems (e.g., integral component of membrane and plasma membrane) were significantly enriched in GO terms (Fig. 3a). Moreover, KEGG enrichment analyses also detected several biological processes about ECM structure and regulation (e.g., ECM-receptor interaction and focal adhesion), and neuromodulation (e.g., glutamatergic synapses and dopaminergic synapse), highlighting their critical role for pregnancy maintenance of *S. schlegelii*. In addition, several novel KEGG pathways were uncovered related to signal transduction, metabolism regulation, including cAMP signaling pathway, thyroid hormone signaling pathway and protein digestion and absorption (Fig. 3b).

The Identification of Positive Selection Genes for *S. schlegelii* Genome

To detect evolutionary drivers for ovoviviparous specialization of *S. schlegelii*, positive selection analysis for 2,242 single copy orthologous genes was implemented in PAML. According to the branch-site model results, a total of 164 genes were subjected to strong positive selection (Table S5). The GO enrichment analysis for PSGs revealed significant involvement in processes mainly related to mitochondrial function (e.g., mitochondrial translational termination, mitochondrial translation and regulation of mitochondrial mRNA stability), cilium structure and movement function (e.g., motile cilium, cilium movement, cilium movement involved in cell motility and dynein heavy chain binding), metabolic pathways (e.g., malate metabolic process, fatty acid beta-oxidation, coenzyme A biosynthetic process and chondroitin sulfate catabolic process) and angiogenesis (e.g., vascular endothelial growth factor-activated receptor activity and vascular endothelial growth factor signaling pathway), additional enriched GO terms included peroxisome (e.g., protein targeting to peroxisome, protein import into peroxisome matrix) and telomere (e.g., establishment of protein localization to telomere, nuclear telomere cap complex) related biological processes (Fig. 4a). Moreover, significantly enriched KEGG pathways, were also predominantly associated with metabolic pathways including carbon metabolism, pyruvate metabolism, and sphingolipid metabolism. Additionally, several vital pathways including peroxisome, homologous recombination, RNA transport and mTOR signaling pathway were also identified (Fig. 4b).

Expression Profiles of Candidate Functional Genes

To investigate the functional relevance of ECM structure and regulation in *S. schlegelii* reproduction, we analyzed expression dynamics of 50 expanded ECM-associated genes across unfertilized (V ovary stage) and gestational ovaries (optic vesicle and pigmentation stages). Distinct expression profiles were identified using RNA-seq datasets (Fig. 5a**,** Table S6). A cohort of genes, including *col4a3*, *col4a4*, *col4a5*, *col11a2*, *cldn10*, and *slit2*, exhibited initial upregulation at the optic vesicle stage followed by decreased expression during pigmentation. Conversely, genes such as

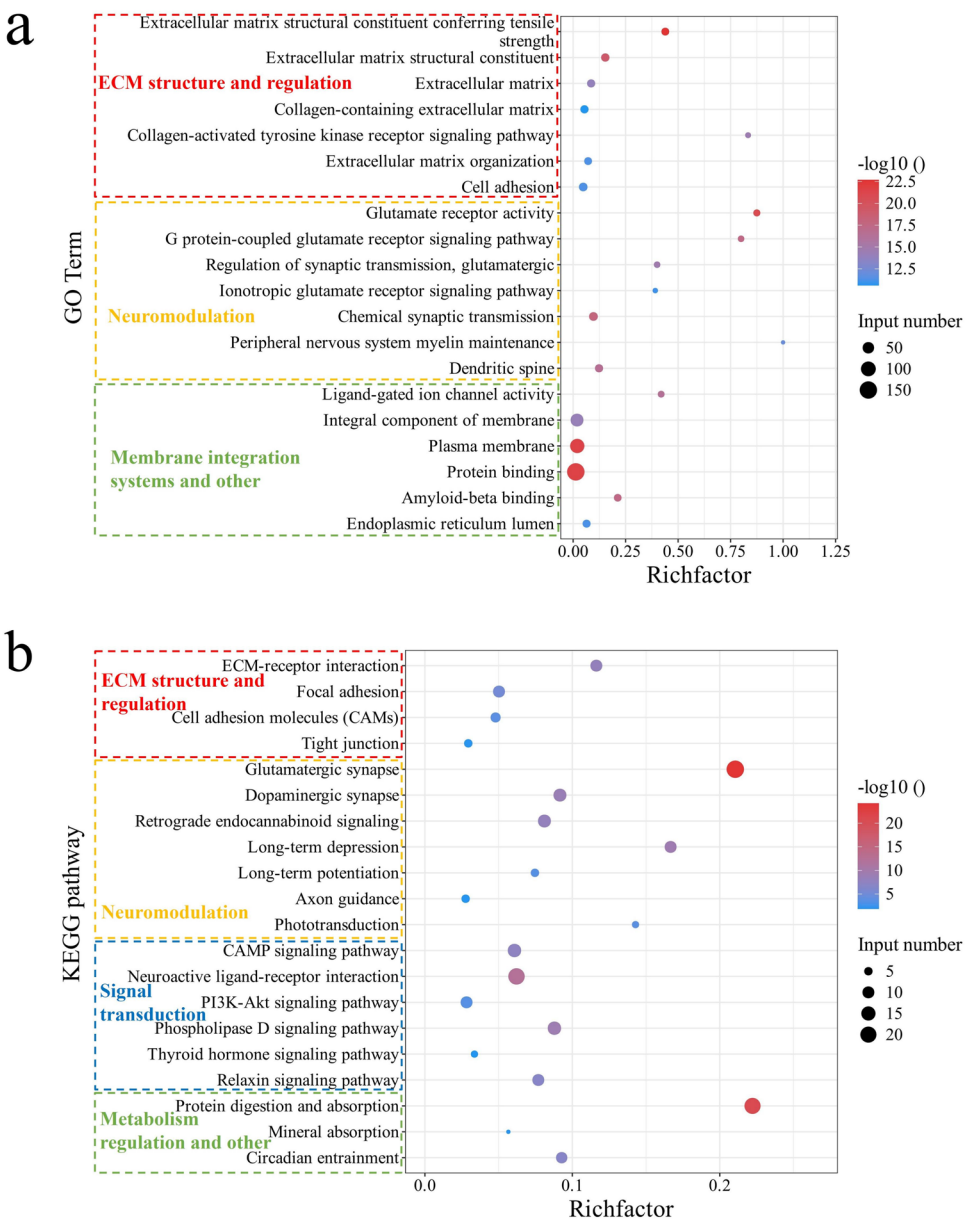

Fig. 3 **(a)** GO enrichment analysis of the significantly expanded gene families, **(b)** KEGG enrichment analysis of the significantly expanded gene families

col5a3, *col4a1*, *col4a2*, *col4a4*, *nectin3*, *rhbdf1*, and *nxpe3* demonstrated sustained high expression throughout gestation. Biphasic regulation was observed for *apba1*, *cntnap5*, *col5a1*, *col11a2*, and *rhbdf1*, characterized by transient downregulation at the optic vesicle stage and subsequent reactivation during pigmentation. A subset of genes (*cntnap4*, *col1fa1*, *nxpe3*, *col4a6*, *apba1*, *col4a5*, and *col11a1*) displayed progressive downregulation post-fertilization.

Furthermore, transcriptional profiling of 29 positively selected angiogenesis-related genes revealed stage-specific expression dynamics (Fig. 5b**,** Table S7). Critical vascular regulators, including *gnpnat1*, *flt1*, *kdr* and *cyldl* genes, showed persistently elevated expression during both optic vesicle and pigmentation stages, underscoring their essential roles in gestational adaptation. Genes such as *vmn2r1*, *rbck1*, *plxdc1*, *peak1*, and *cspg4* exhibited the expression pattern with initial elevation followed by downregulation. Conversely, *tekt1*, *dnajc10*, *map3k9*, *rsph9*, *fzd4*, *myo3a*, and *efhc2* demonstrated early-phase upregulation at the optic vesicle stage followed by progressive downregulation. Additionally, the expression pattern of *tert*, *epm2a*, *ptges2*, *gfm2* and *tdgf1* were downregulated with moderate or high extent following fertilization. The RNA-seq data supporting these analyses have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1309490, and the

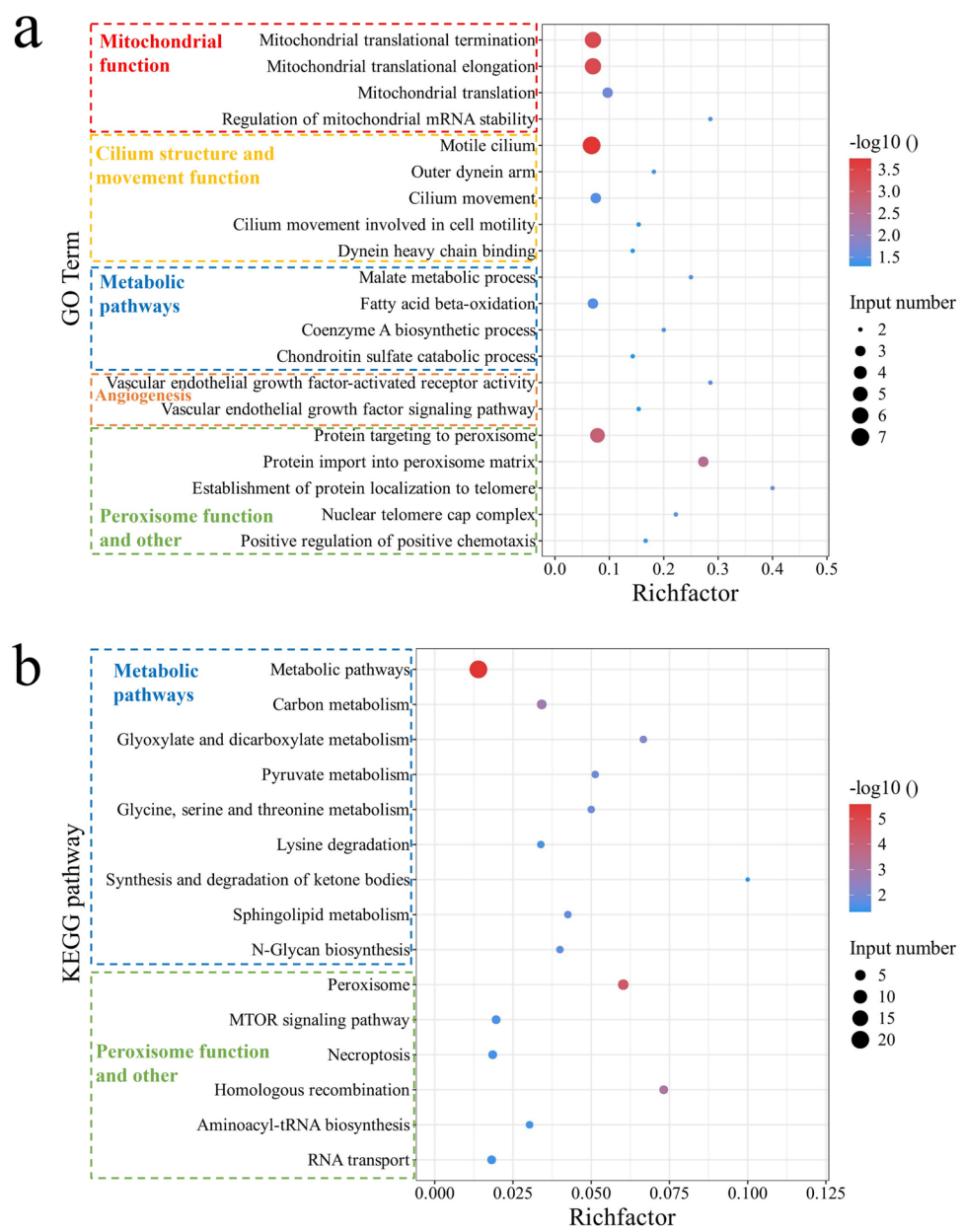

Fig. 4 **(a)** GO enrichment analysis of PSGs in *S. schlegelii*, **(b)** KEGG enrichment analysis of PSGs in *S. schlegelii*

individual SRA accession numbers will be available upon release.

Discussion

As a commercially crucial ovoviviparous aquaculture species in China, the *S. schlegelii* exhibits a distinctive reproductive strategy characterized by internal fertilization and prolonged embryonic development culminating in live birth. Pregnancy maintenance represents a critical biological process for artificial breeding programs, where mechanistic understanding directly informs reproductive efficiency optimization, germplasm resource conservation, and selective breeding strategies (Holt & Pickard 1999; Lima et al. 2010; Thatcher et al. 2002). To elucidate these adaptations, we conducted comparative genomic analysis between *S. schlegelii* and representative fish species, systematically identifying lineage-specific gene families, expanded or contracted gene families, as well as positively selected genes for *S. schlegelii*. These gene resources reflected adaptive evolutionary changes necessary to support the specialized reproductive mode of ovoviviparity. In addition, the candidate functional genes were determined using the RNA-seq datasets derived from stage V ovary, optic vesicle stage and pigmentation stage for ovarian stroma tissue. Overall, this study provided novel insights

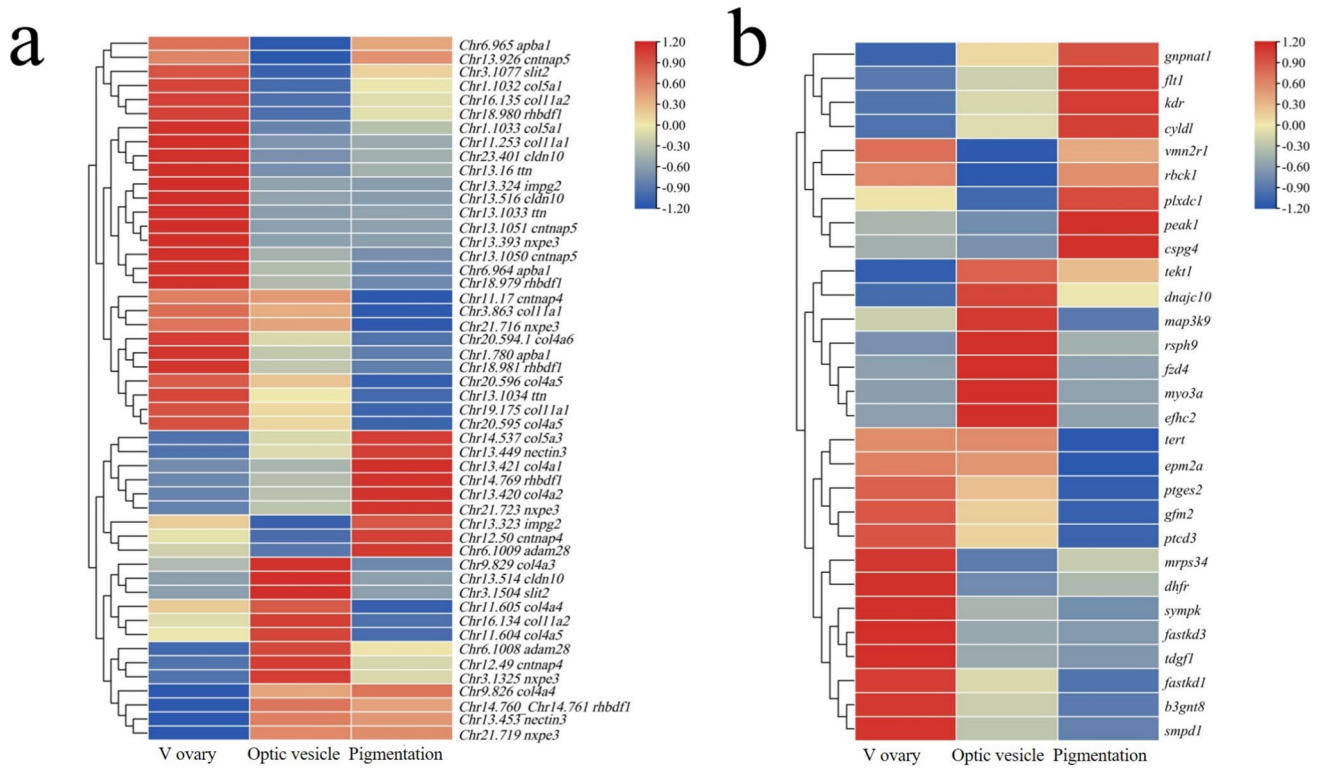

Fig. 5 Expression profiles of expanded ECM-associated genes **(a)** and positively selected angiogenesis-related genes **(b)** under unfertilized (V ovary stage) and gestational ovaries (optic vesicle and pigmentation stages) for *S. schlegelii*

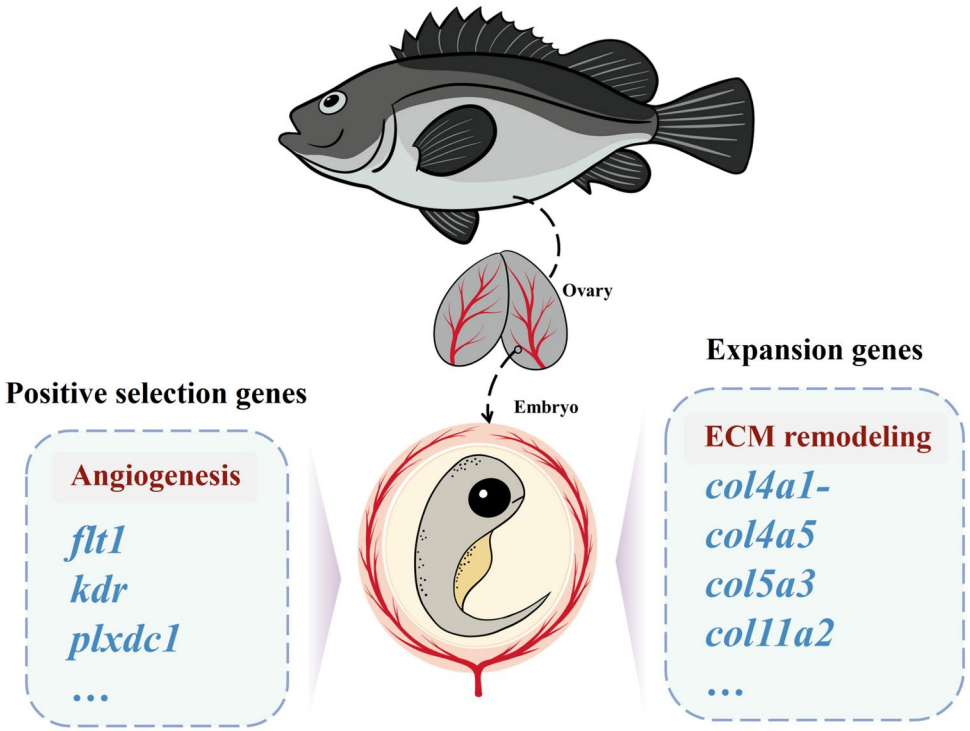

Fig. 6 Mechanistic model of pregnancy maintenance in *S. schlegelii*, derived from integrated genomic and transcriptomic analyses. Positively selected genes (e.g., *flt1*, *kdr*, *plxdc1*) are associated with angiogenesis, promoting vascular remodeling and maternal nutrient supply. Expanded ECM-related gene families (e.g., *col4a1-col4a5*, *col5a3*, *col11a2*) contribute to ECM remodeling and ovarian tissue support. Together, these adaptations highlight coordinated angiogenesis and ECM remodeling as key mechanisms for maintaining the ovarian microenvironment during intraovarian gestation

into the genomic adaptation associated with ovoviviparous reproduction in *S. schlegelii* and presented a putative schematic diagram displaying the functionally responsive genes involved in pregnancy maintenance mechanisms, serving as a roadmap for future investigations into the pregnancy maintenance mechanisms (Fig. 6).

In our study, gene families clustering analysis of *S. schlegelii* in comparison with *O. latipes*, *D. rerio*, *P. reticulata*, *R. typus* revealed 374 lineage-specific gene families in *S. schlegelii* (Fig. 1). These genomic innovations showed significant functional enrichment in four core biological processes, including immune regulation, cellular signaling, metabolic control, and developmental biology, as determined through GO enrichment analysis (Table S2). Additionally, KEGG analysis identified significant enrichment in neural synaptic transmission, hormone signaling pathways, and immune system networks (Table S2). In pregnant mammals, the successful maintenance of pregnancy depends on precisely regulated immunological adaptations (Aagaard-Tillery et al. 2006; Adar et al. 2015). During this period, the maternal immune system must engage in a fine balancing act: maintaining tolerance to the fetal allograft while preserving essential innate and adaptive immune functions to defend against microbial threats (Aagaard-Tillery et al. 2006; PrabhuDas et al. 2015). Beyond immunological tolerance, the maternal–fetal interface necessitates the bidirectional transfer of nutrients and molecular signals to support fetal growth and maternal adaptation. This complex interplay relies on the integration of several tightly regulated biological systems, including cellular signaling, which governs communication between maternal and fetal tissues (Gude et al. 2004). Metabolic control, which ensures energy allocation and nutrient availability tailored to fetal demands (Napso et al. 2018). In addition, synaptic transmission plays a role in modulating maternal behavior and neuroendocrine adaptation during gestation (Hoekzema et al. 2017), and several hormone signaling pathways mediated by progesterone and human chorionic gonadotropin, orchestrate uterine quiescence, placental development, and endocrine regulation were critical for gestational success (Costa 2016).

Our phylogenetic analyses estimated the divergence time between *S. schlegelii* and *O. mykiss* at approximately 231 million years ago (Fig. 2a). This estimation was concordant with the previously reported range of 176–264 MYA, derived from various molecular clock approaches for *S. schlegelii*, *O. mykiss* and other species (Li et al. 2018; Rabosky et al. 2018), supporting the reliability of our divergence time estimation. Moreover, phylogenetic topology reveals *S. schlegelii* demonstrated the closest evolutionary affinity to oviparous taxa among the six examined ovoviviparous species, which further forms a separate clade in phylogenetic tree. This pattern suggests that ovoviviparity in *S. schlegelii* likely evolved secondarily from oviparous ancestors, retaining genomic and reproductive features reminiscent of oviparous lineages such as *O. latipes* and *D. rerio*. Notably, *S. schlegelii* exhibits an exceptionally high fecundity, with females carrying several hundred thousand embryos during gestation, a capacity comparable to highly fecund oviparous fishes (Linhart et al. 1995; Mori et al. 2003). At the same time, embryos receive substantial maternal provisioning during intraovarian gestation, reflected by a matrotrophy index (MI) of approximately 1.8, which is higher than reported for the four examined ovoviviparous poeciliid species(Boehlert et al. 1986; Pollux et al. 2009; Van Kruistum et al., 2021). This unusual combination of extremely high fecundity and enhanced maternal nutrient transfer underscores *S. schlegelii* as a unique transitional model that simultaneously retains oviparous-like reproductive capacity while also evolving viviparity-like maternal investment strategies. Comparative genomic analyses incorporating additional oviparous lineages with annotated genomes, together with broader sampling across diverse ovoviviparous and viviparous fishes, will be crucial to determine whether the genomic features identified in *S. schlegelii* reflect retained oviparous ancestry, unique lineage-specific adaptations, or convergent mechanisms associated with repeated origins of viviparity in teleosts.

Gene families that under expansion and contraction represent fundamental drivers of adaptive evolution, enabling functional diversification through genomic specialization. The expanded gene families further play a pivotal role in enhancing species'adaptability by providing genetic diversity and facilitating the development of new functions that contribute to evolutionary success (R. Wang et al. 2025). In *S. schlegelii*, the 73 significantly expanded gene families (comprising 276 genes) demonstrates significantly enriched functions across four critical adaptation modules: ECM structure and regulation, neuromodulation, transmembrane transport, and membrane integration (Fig. 3), suggesting that these biological processes are essential for the successful maintenance of pregnancy in *S. schlegelii*. Multiple glutamate signaling processes including glutamate receptor activity, G protein-coupled glutamate receptor signaling pathway and ionotropic glutamate receptor signaling pathway were detected in the neuromodulation processes, which play a critical role in regulating neuronal activity and synaptic plasticity (Niswender & Conn 2010; Reiner & Levitz 2018; Traynelis et al. 2010). In the context of pregnancy, these glutamate signaling processes pathways may influence maternal behaviors (Salmaso et al. 2011), hormonal regulation (Tsesis et al. 2013), and communication between maternal and fetal tissues (Wu et al. 2015; Zhu et al. 2015), which all are necessary for stable pregnancy process. As an illustrative example, collagen type I alpha 1 (*COL1A1*), a downstream target

of glutamine, could activate the PI3K-AKT pathway in HTR-8/SVneo cells, thereby promoting trophoblast invasion by upregulating *COL1A1* expression, a process that is critical for facilitating adequate embryonic nutrition. (J. Shi et al. 2024). These results collectively emphasize the vital roles of neuromodulation processes in pregnancy maintenance through several approaches.

Notably, significant expansion was observed for ECM-related genes, particularly in collagen types IV (*col4a1-col4a5*), V (*col5a3*), and XI (*col11a2*), indicating the conserved evolutionary role in pregnancy mechanisms (Fig. 6). Notably, expression profiling revealed that these genes exhibit upregulated expression following the onset of pregnancy, further supporting their functional relevance in gestational tissue remodeling and maternal–fetal interface integrity. During the establishment of a successful pregnancy for human, the endometrium undergoes significant changes, including the breakdown and remodeling of the ECM, with decidualization and trophoblast invasion closely associated with extensive ECM remodeling, where collagen and its fragments play a critical role at the maternal–fetal interface (J.-W. Shi et al. 2020). As primary structural components of the ECM scaffold, collagens consisting of 29 members play a critical role in regulating cellular behavior and maintaining the structural integrity of tissues, mainly through forming supramolecular structures such as fibrils (types I and III), networks (type IV), or beaded filaments (type VI) (Ricard-Blum 2011; Sorushanova et al. 2019). Additionally, the high expression of collagen at the maternal–fetal interface is primarily produced by decidual stromal cells (DSCs) and trophoblasts (Fu et al. 2014, 2017). Collagen type IV (col-IV), a non-fibrillar basement membrane constituent, provides structural support while modulating cell adhesion, migration, and survival (Schwarzbauer 1999). The col-IV facilitates trophoblast invasion and ECM remodeling at the implantation site, with its selective upregulation during decidualization and its unique structural presence in the placenta (Oefner et al. 2015). Our findings parallel these mechanisms, demonstrating the significance of col-IV in ECM remodeling and cell migration during the early development of *S. schlegelii*. Collagen type V (col-V) plays a crucial role in the initiation of collagen fibril assembly and regulates fibril number, diameter, and overall extracellular matrix organization (Wenstrup et al. 2004). During early pregnancy in mice, col-V is primarily localized in non-decidualized regions and around blood vessels, first appearing in the mature decidua and predecidua on day 7 as a network of thin fibers; throughout all stages, strong expression is consistently observed between smooth muscle cell bundles in the myometrium (Spiess et al. 2007). Studies on col-V in human decidual tissue have suggested its involvement in maintaining pregnancy, as decreased expression has been associated with spontaneous abortion(Iwahashi & Nakano 1998). Consistently, col-V has been detected throughout all layers of the fetal membranes in humans, though with varying intensities (Malak et al., 1993), further supporting its potential role in fetal-maternal interface stability. Collagen type XI, a minor fibrillar collagen, is also distributed in the placenta, can regulate fibrillogenesis by maintaining the spacing and diameter of collagen type II fibrils and works as a nucleator for the fibrillogenesis of collagen type I and II (Fernandes et al. 2007; Kadler et al. 2008; Vaughan-Thomas et al. 2001). However, its specific role in pregnancy maintenance remains unclear and warrants further investigation.

Positive selection analysis identified 164 genes under strong selective pressure, significantly enriched in mitochondrial function, cilium movement, metabolic pathways, angiogenesis, peroxisomal activity, and telomere maintenance. The positive selection of mitochondrial and metabolic genes likely correlates with the high energetic demands associated with sustaining embryonic development within maternal tissues (Burton & Fowden 2012; Ellison 2003; Ghosh et al. 2023). Additionally, genes involved in cilium structure and movement may contribute to critical embryonic developmental processes, such as cell migration and differentiation (Amack 2022; Yanardag & Pugacheva 2021). The positive selection of angiogenesis-related genes, especially within the VEGF signaling pathway, further emphasizes the adaptive significance of vascular remodeling to ensure adequate oxygen and nutrient delivery during pregnancy (Harris & Aplin 2007; Osol & Mandala 2009). Furthermore, expression profiling of angiogenesis-related genes revealed distinct stage-specific patterns during gestation. Genes upregulated during the pigmentation stage, including *flt1*, *kdr*, *plxdc1*, *gnpnat1*, and *cspg4*, are primarily associated with endothelial cell signaling, extracellular matrix interactions, and immune modulation (Barili et al. 2023; Beaty et al. 2007; Harrer et al. 2022; Shibuya 2001, 2006; Waltenberger et al. 1996), highlighting their roles in ovarian vascular remodeling (Fig. 6). Conversely, genes elevated during the optic vesicle stage, such as *fzd4* and *map3k9*, are implicated in cellular proliferation, oxidative stress response, and signal transduction pathways (Skronska-Wasek et al. 2017; Y. Wang et al. 2018; Xia et al. 2018), suggesting critical molecular events necessary for embryonic vascular support and development. Collectively, these results underscore that positively selected genes exhibit finely tuned temporal expression profiles aligned with specific physiological demands during ovoviviparous pregnancy. This temporal regulation of angiogenesis-related PSGs, particularly

those involved in ECM interaction and endothelial signaling, reflects a stage-specific remodeling process that likely parallels essential reproductive adaptations seen in mammals. In humans, successful pregnancy depends on spiral artery remodeling, a process involving coordinated ECM degradation, immune cell recruitment, and vascular reconfiguration to support maternal–fetal exchange (Harris & Aplin 2007; Robson et al. 2012). Although ovoviviparous teleosts lack a uterus, the upregulation and positive selection of genes such as *flt1*, *kdr*, *cspg4*, and *plxdc1* suggest a functionally analogous form of ovarian vascular remodeling during internal gestation (Fig. 6). This remodeling may facilitate enhanced oxygen and nutrient delivery to developing embryos within the ovarian environment. The evolutionary pressure acting on these genes highlights their adaptive significance in meeting the physiological demands of sustained embryo retention and growth in ovoviviparous species.

While this study provides preliminary insights into the genomic basis underlying ovoviviparity in *S. schlegelii*, functional validation remains incomplete. Future research could employ targeted experimental approaches to test the roles of candidate genes. For instance, CRISPR/Cas9-mediated knockout or knockdown of angiogenesis-related genes (e.g., *flt1*, *kdr*) may help clarify their contribution to ovarian vascular remodeling, while in situ hybridization or immunohistochemistry of ECM-associated genes (e.g., *col4a1-col4a5*) could validate their spatial expression patterns in ovarian stroma. Furthermore, integrating these molecular approaches with physiological measurement such as vascular density and ECM deposition would provide stronger functional evidence. Expanding comparative genomic analyses to additional ovoviviparous and viviparous teleost species will further enhance our understanding of the evolutionary mechanisms driving reproductive diversity in fish.

Conclusions

In this study, by performing comparative genomic analysis between *S. schlegelii* and other oviparous and ovoviviparous fish, we identified 374 lineage-specific and 73 significant expanded gene families. In addition, 164 genes were detected under strong positive selection. These genes are of great interest for understanding the genetic basis underlying pregnancy maintenance in *S. schlegelii*. Combined with transcriptomic analyses, the expression levels of candidate genes involved in ECM remodeling and angiogenesis were significantly changed following pregnancy in *S. schlegelii*, suggesting their potential roles in supporting gestational processes. Together, these findings provide valuable insights into key molecular mechanisms underlying pregnancy maintenance in *S. schlegelii*.

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Data Availability The RNA-seq data supporting these analyses have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1309490, and the individual SRA accession numbers will be available upon release.

Declarations

Conflict of interest The authors declare no competing interests.

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