

Characterization of a low-density lipoprotein receptor, *Lrp13*, in Chinese tongue sole (*Cynoglossus semilaevis*) and medaka (*Oryzias latipes*)

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Abstract As an important economic marine species cultured in China, Chinese tongue sole (*Cynoglossus semilaevis*) has interested us due to its sexual dimorphism and ZW/ZZ sex determination system. In a previous study, *dmrt1* was identified as a dosage-dependent male-determining gene. In the present study, a female-specific expressed gene, *cse0440*, initially annotated as *lrp1b-like*, was identified from chromosome W of *C. semilaevis*. In view of the differences between *cse0440* and *lrp1b* in

terms of expression pattern, a phylogenetic analysis containing 85 LRP proteins was constructed and provided an evidence to re-annotate *cse0440* as *cseLRP13*. In addition, two orthologues of *cseLRP13* were separately identified from W and Z chromosomes: *cseLRP13-W* and *cseLRP13-Z*. The subsequent multiple sequence alignment and syntenic arrangements of LRP13 in *C. semilaevis*, Japanese medaka (*Oryzias latipes*), large yellow croaker (*Larimichthys crocea*), striped bass (*Morone saxatilis*), white perch (*Morone americana*) and Fugu rubripes (*Takifugu rubripes*) further supported this re-annotation. RT-PCR and in situ hybridization revealed that *cselrp13* was exclusively expressed in the oocytes and follicles of ovaries. These results suggested that *lrp13* may play important roles in female reproduction. In future, with the advancement of micromanipulation in flatfish, the detailed function of two *lrp13* orthologues in *C. semilaevis* will be elucidated.

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Introduction

The mechanism of sex determination has been continuously studied for many decades from simple eukaryotes to mammals. Higher vertebrates, such as mammals and birds, exhibit genetic sex determination (GSD), in which sex is controlled by genetic factors on sex chromosomes or autosomes (Sinclair et al. 1990; Chue and Smith 2011;

Smith et al. 2009). Meanwhile, in lower vertebrates, including fish, sex determination could range from GSD to environmental sex determination (ESD), where environmental factors include temperature, pH, density and social interactions (Penman and Piferrer 2008; Piferrer and Guiguen 2008).

In terms of genetic factors, since the revelation of the first male sex-determining gene (SRY/Sry) in mammals (Sinclair et al. 1990), other candidates for male sex determination were sequentially discovered, including *Dmy* in medaka (*Oryzias latipes*) (Matsuda et al. 2002), *Dmrt1* in birds (Smith et al. 2009), *amhy* in the Patagonian pejerrey (*Odontesthes hatcheri*) (Hattori et al. 2012) and *gsdf* in medaka (*Oryzias latipes*) (Myosho et al. 2012). Meanwhile, few female sex-determining genes were found on the W chromosome except the paralogue of *Dmrt1-Dmw* in the South African clawed frog (*Xenopus laevis*) (Yoshimoto et al. 2008).

Chinese tongue sole (*Cynoglossus semilaevis*), an important economic marine species cultured in China, has become a wonderful material for sex determination research owing to its sexual dimorphism, abundant genomic resources and female heterogametic (ZW/ZZ) sex determination system (Song et al. 2012; Sha et al. 2010; Shao et al. 2010; Chen et al. 2007; Zhou et al. 2005). Recently, based on the whole-genome sequence of *C. semilaevis*, doublesex and mab-3-related transcription factor 1 (*dmrt1*) located on Z chromosome has been identified as a dosage-dependent male-determining gene (Chen et al. 2014). Meanwhile, 317 predicted functional protein-coding genes were identified on female specific chromosome and several of them were only detected in female tissues or gonads (Chen et al. 2014). In the present study, a W chromosome-localized gene, CSE_GLEAN_10000440 (cse0440), initially annotated as low-density lipoprotein receptor-related protein 1B-like (LRP1B-like) in the NCBI database, was chosen as our research target.

LRP1B, part of the LRP family, was originally described as a putative tumour suppressor in lung cancer cells (Cam et al. 2004), and its silence at a genetic or epigenetic level was a key symbol in primary oesophageal tumours (Sonoda et al. 2004). In addition, LRP1B functioned as a negative regulator of urokinase plasminogen activator receptor (uPAR) regeneration and cell migration (Li et al. 2002). In mammals, *lrp1b* is widely expressed in the brain, thyroid, salivary gland, adrenal gland and testis (Liu et al. 2001; Li et al. 2005). While, in *C. semilaevis*, *cse0440/lrp1b-like* was only detected in the ovary, which implied that *cse0440* may be not the orthologue of *lrp1b*.

Apart from LRP1B, there are other prominent members of the LRP family: LRP1–6, LRP8 and LRP10–12 (Strickland et al. 2002; Bajari et al. 2005; Battle et al. 2003). Recently, the report of a vertebrate lipoprotein receptor, LRP13, attracted our attention due to its predominant expression pattern in the ovaries of striped bass (*Morone saxatilis*), white perch (*Morone americana*) (Reading et al. 2014) and cutthroat trout (*Oncorhynchus clarkii*) (Mushirobira et al. 2015).

In the present study, we conducted a phylogenetic analysis for different LRP genes and re-annotated cse0440 as cseLRP13. At the same time, two orthologues of cseLRP13 were separately identified from chromosomes W and Z: cseLRP13-W and cseLRP13-Z. Meanwhile, LRP13 genes from other species, including medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), Atlantic salmon (*Salmo salar*), house mouse (*Mus musculus*), cattle (*Bos taurus*), chicken (*Gallus gallus*), Fugu rubripes (*Takifugu rubripes*) and large yellow croaker (*Larimichthys crocea*), were also identified. The multiple sequence alignment and syntenic arrangements of LRP13 in several fish species further supported this re-annotation. The expression patterns of *cseLRP13*, *cseLRP1b* and *O. latipes lrp13* (*olalrp13*) were analysed by RT-PCR and in situ hybridization.

Materials and methods

Experimental fish

C. semilaevis was maintained from Haiyang Yellow Sea Aquatic Product Co., Ltd. The orange strain of *O. latipes* (kindly gifted from Institute of Hydrobiology, Chinese Academy of Science) was cultivated and bred in an aquarium under an artificial 14 h light/10 h dark photoperiod at 28 °C at the Yellow Sea Fisheries Research Institute, Chinese Fishery Science Academy.

The sequence confirmation and analysis

Based on the cse0440 complementary DNA (cDNA) sequence in NCBI database, primers (cse0440_GSP1, cse0440_NGSP1, cse0440_GSP2, cse0440_NGSP2) were designed to verify the sequence using a SMARTer RACE 5'/3' Kit. Furthermore, the deduced proteins were submitted to Simple Modular Architecture Research Tool (<http://smart.embl-heidelberg.de/>) to search conserved domains.

The phylogenetic analysis of LRP proteins

To identify the genuine orthologue of *cse0440*, a phylogenetic analysis was conducted using MEGA 7 (Kumar et al. 2016). Briefly, a BLASTp search was carried out to look for sequences homologous to *cse0440* protein (XP_008333657.2). A set of homologues of *cse0440* were selected and identified based on the information of max score, total score, query coverage and max identity. The other homologues of LRP family were also included. The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model. All positions with less than 95% site coverage were eliminated. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

The multiple sequence alignment and syntenic arrangements of several fish species LRP13

To further confirm the LRP13 homology in fish species, LRP13 proteins from eight fish species (*C. semilaevis*, *O. latipes*, *M. saxatilis*, *M. americana*, *D. rerio*, *T. rubripes*, *L. crocea* and *O. clarkii*) were used for the multiple sequence alignment by DNAMAN software. Additionally, based on the information derived from NCBI database, syntenic arrangements of LRP13 genes from five fish species, including *C. semilaevis*, *O. latipes*, *D. rerio*, *L. crocea* and *T. rubripes*, were analysed.

The expression pattern analysis of *cselrp13*, *cselrp1b* and *olalrp13*

To analyse the expression pattern of *cselrp13* and *cselrp1b*, ten tissues of 2-year-old *C. semilaevis* (skin, muscle, liver, kidney, intestine, heart, gill, brain, spleen and gonad) and seven tissues of 6-month-old *O. latipes* (intestine, gill, liver, muscle, brain, eye and gonad) were sampled from three female and three male individuals.

The total RNA was extracted using an RNAfast200 Kit (Fastagen, China) and reverse transcribed into cDNA using FastQuant RT Super Mix (Tiangen, China). The expression patterns were examined by semi-

quantitative PCR with the specific primers listed in Table 1. The primers *cseLRP13-F/cseLRP13-R*, *cseLRP1B-F/cseLRP1B-R* and *olaLRP13-F/olaLRP13-R* were designed to amplify a specific fragment of 124, 119 and 163 bp, respectively. The amplified products were confirmed by sequencing. β -Actin and *18 s* were used as the internal reference genes of *C. semilaevis* and *O. latipes*.

In situ hybridization

To examine the spatial location of *cselrp13* in *C. semilaevis*, a 600-bp fragment was amplified by primers *cseLRP13+E*, *cseLRP13-H* and subcloned into plasmid pBluescript II SK+. T7 and T3 RNA polymerases were separately used for synthesis of the antisense and sense probes with DIG RNA Labelling Mix (Roche, USA). The in situ hybridization was performed at different *C. semilaevis* developmental stages (44, 68, 83, 150 and 311 days) as described in the previous method (Chen et al. 2014) with slight modifications. Briefly, the gonad parts were fixed in 4% paraformaldehyde overnight and stored in methanol. The treated tissues were then embedded in paraffin, sectioned at 5 μ m and subjected to in situ hybridization. Subsequently, the section slides were deparaffinized, hydrated, treated with proteinase K (10 mg/ml) and then hybridized by sense or antisense probes at 65 °C for overnight. After washing with SSC solutions, slides were treated with alkaline phosphatase conjugated anti-DIG antibody (Roche Applied Science, Germany) overnight at 4 °C. Finally, the samples stained with NBT-BCIP solution were observed and captured under a Nikon Eclipse 80i microscope.

Results

New annotation of *cse0440*

Combined with the results of high-throughput sequencing and RACE confirmation, a full-length *C. semilaevis* *cse0440* mRNA sequence was obtained. The transcript contains a 22-bp 5'UTR, a 3981-bp open reading frame and a 52-bp 3'-untranslated region (GenBank, XM_008335435.2). When analysed by SMART, a putative signal peptide and a transmembrane region are separately predicted at the N-term and C-term. In addition, several typical features of other LRP family members, including 11 low-density lipoprotein receptor

Table 1 Primers used in this study

Primer name	Sequence (5'-3')	Primer information
cse0440_GSP1	CATCTAGCGAGGACCACCTGATGCTC	5'RACE amplification
cse0440_NGSP1	CTGACCCAGTAGACCCTCTTCTCCTTC	5'RACE amplification
cse0440_GSP2	GGAGCCCTTCTGTTGGCATCGA	3'RACE amplification
cse0440_NGSP2	GCCACAGCTTGAGTGTGTTGTCTCTCT	3'RACE amplification
cseLRP13-F	TGCTTTAGGGATCATTGGAT	Semi-quantity RT-PCR
cseLRP13-R	TGTTCTGTTGCTGCTGTCTC	Semi-quantity RT-PCR
cseLRP1B-F	ACCCTCTGATGGGAATG	Semi-quantity RT-PCR
cseLRP1B-R	AGTGGGCTGCTCTGTC	Semi-quantity RT-PCR
olaLRP13-F	TGAAGCGGACTGATA	Semi-quantity RT-PCR
olaLRP13-R	GCTGTAGGGACTGTAAT	Semi-quantity RT-PCR
cseactin-F	TTCCAGCCTTCCTTCCTT	Semi-quantity RT-PCR
cseactin-R	TACCTCCAGACAGCACAG	Semi-quantity RT-PCR
ola18s-F	TCCGATAACGAACGAGAC	Semi-quantity RT-PCR
ola18s-R	GGACATCTAAGGGCATCA	Semi-quantity RT-PCR

domain class A (LDLa), 8 low-density lipoprotein receptor YWTD domains (LY), 3 epidermal growth factor-like domain (EGF) and 1 calcium-binding EGF-like domain (EGF_CA), are observed. Although cse0440 is annotated as LRP1B-like in the NCBI database, it has obvious differences in expression patterns that indicated it was not a genuine LRP1B-like gene. BLASTp was employed, and several LRP proteins showing up to 50% identity with cse0440 were selected. *M. Americana* LRP13 (KF387534.1) and *O. clarkii* LRP13 (KR188876.1), which exhibited similar expression patterns with cse0440, were also listed. With the addition of the other homologues of LRP proteins, a total of 85 LRP proteins were used for the construction of phylogenetic tree (Fig. 1), which contained 12 subtrees: LRP1B, LRP1–6, LRP8, LRP10–12 and LRP13. Our research targets, cse0440/XP_00833657.2 and its homologue in *O. latipes*-ola0440/XP_011478083.1 both belonged to the subtree of LRP13, but not that of LRP1B. In addition, the true LRP1B orthologues of *C. semilaevis* and Japanese medaka (*O. latipes*) were identified in the subtree of LRP1B: XP_016895408.1 and XP_011488326.1. Therefore, the phylogenetic analysis provided strong evidence for the new annotation of cse0440 and ola0440 as cseLRP13 and olaLRP13. In addition, it is worth noting that there is another LRP13 orthologue (LRP2-like XP_008333985.1) in *C. semilaevis* Z chromosome. Thus, two orthologues have been identified in *C. semilaevis*: cseLRP13-W and cseLRP13-Z.

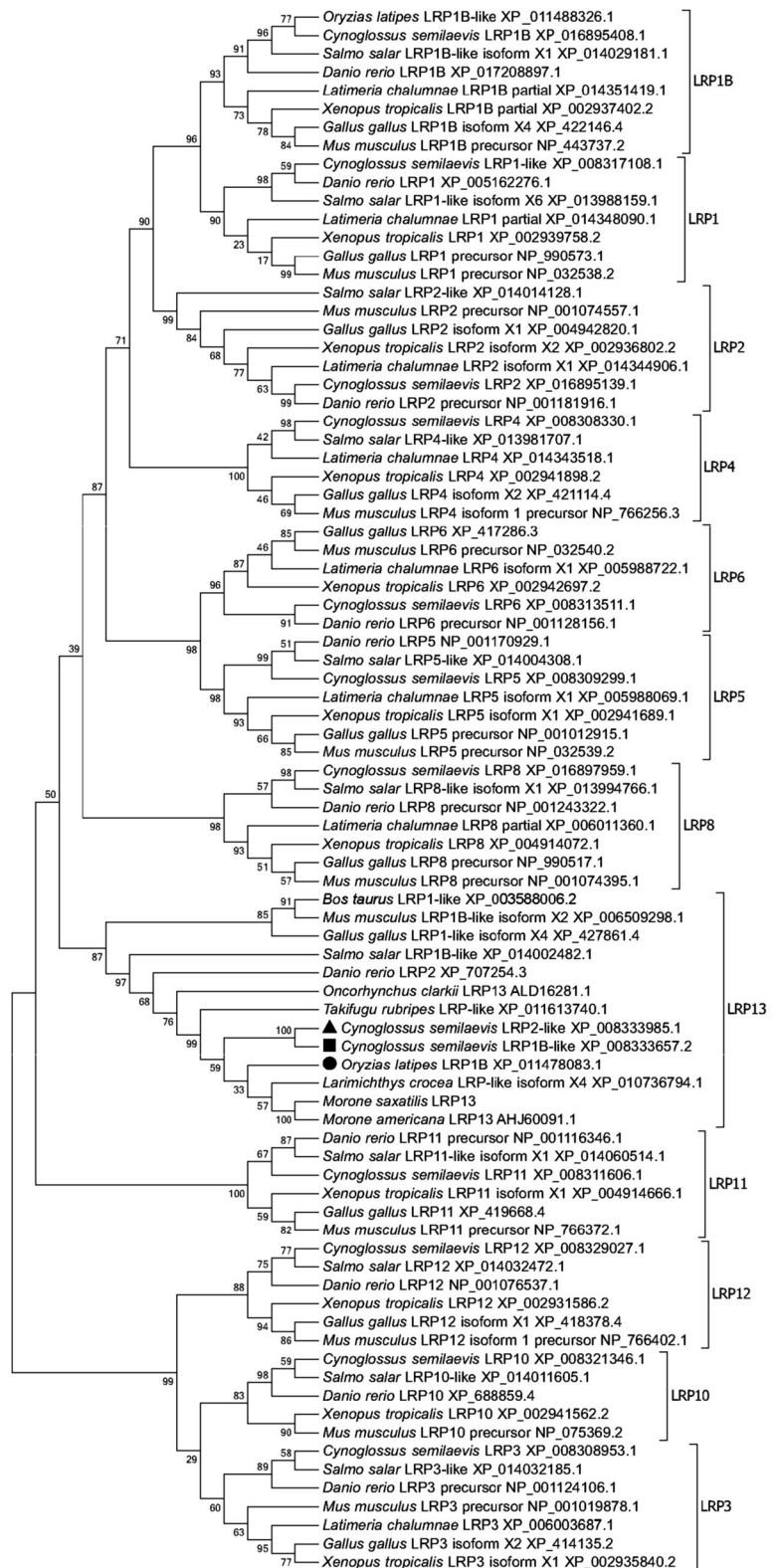
In addition to *C. semilaevis* and *O. latipes*, LRP13 orthologues from other species, including *D. rerio*, *S. salar*, *M. musculus*, *B. taurus*, *G. gallus*, *T. rubripes* and *L. crocea*, were also identified. The multiple sequence alignment (Fig. 2) by nine LRP13 proteins from fish species demonstrated that these fish LRP13 proteins were 65.39% identical. Furthermore, synteny arrangement of LRP13 was compared using *C. semilaevis* chromosome W_12,122,334–12,216,605, *C. semilaevis* chromosome Z_2,019,894–2,071,435, *O. latipes* chromosome 9_25,175,067–25,248,101, *L. crocea* scaffold NW_017608618.1_2,267,866–2,329,685 and *T. rubripes* chromosome 21_5,289,031–5,329,390, which were available from NCBI database. As a result, the arrangements of these LRP13 (Fig. 3) were similar to those of *D. rerio* and Nile tilapia (*Oreochromis niloticus*) (Reading et al. 2014).

Based on the results from phylogenetic analysis, multiple sequence alignment and syntenic arrangement, a lipoprotein receptor-LRP13 was successfully identified from *C. semilaevis* and *O. latipes*.

The expression pattern of *cselrp13* and *cselrp1b* in *C. semilaevis* and *O. latipes*

The different tissue expression patterns of *cselrp13* were analysed by semi-quantitative RT-PCR. Among ten tissues of *C. semilaevis*, *cselrp13* was only detected in the ovary (Fig. 4a). Similarly, *olalrp13* was also only detected in *O. latipes* ovary (Fig. 4b). For *cselrp1b*, its

Fig. 1 Phylogenetic analysis of 85 LRP homologues. A phylogenetic tree involving 85 proteins was constructed using the maximum likelihood method of MEGA7. There were a total of 367 positions in the final dataset. The branches were validated by bootstrap analysis from 1000 replications, which were represented by percentage in branch nodes. Eighty-five LRP homologue proteins used in this analysis are provided as Supplementary file 1. The *black square* and *black triangle* separately indicate two LRP13 homologues of *C. semilaevis*. The *black circle* shows a LRP13 homologue in *O. latipes*



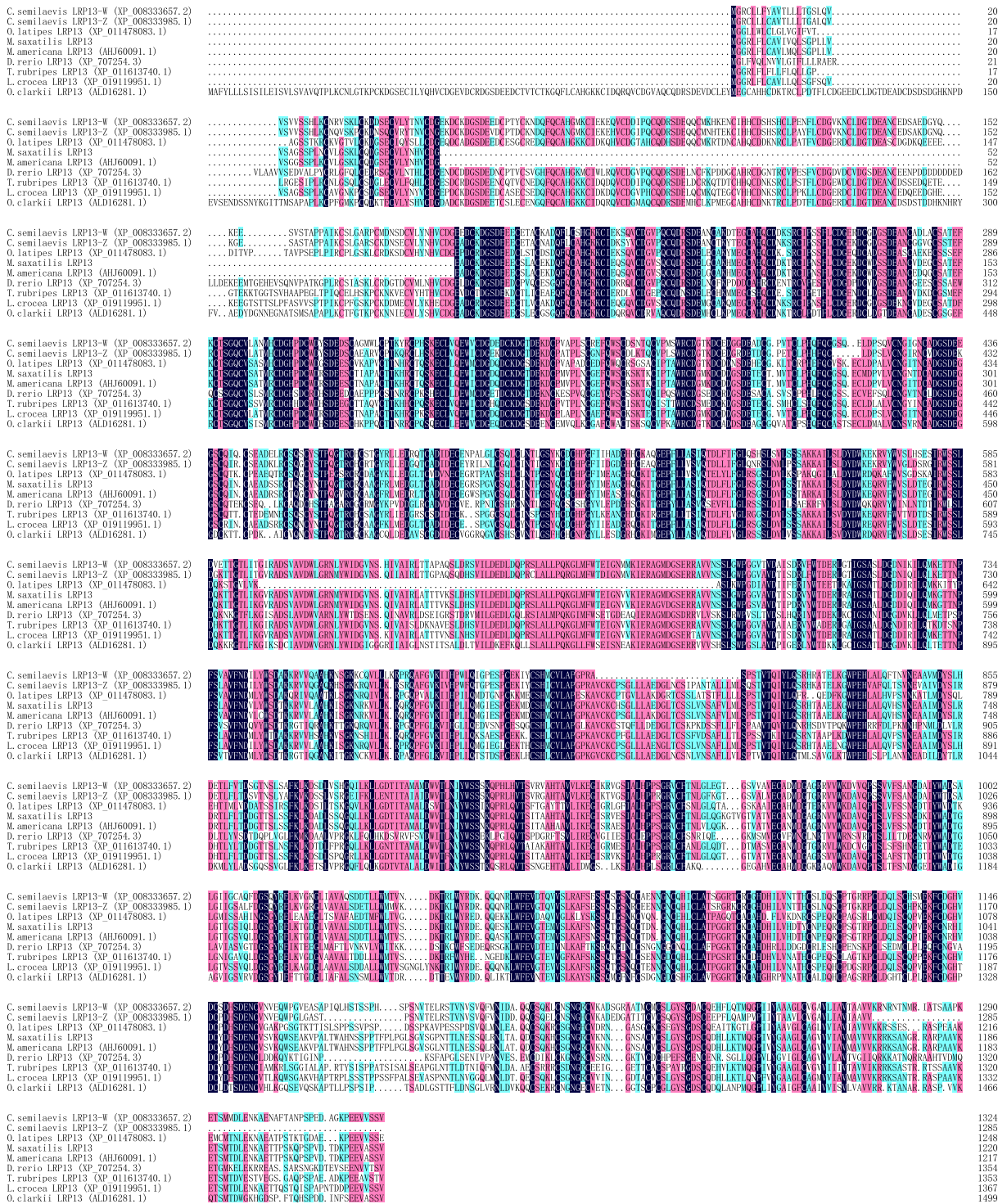


Fig. 2 The multiple sequence alignment of LRP13 proteins in several fish species. Nine LRP13 proteins, including *C. semilaevis* LRP13-W (XP_008333657.2), *C. semilaevis* LRP13-Z (XP_008333985.1), *O. latipes* LRP13 (XP_011478083.1), *M. saxatilis* LRP13, *M. americana* LRP13 (AHJ60091.1), *D. rerio* LRP13 (XP_707254.3), *T. rubripes* LRP13 (XP_011613740.1), *L. crocea* LRP13 (XP_019119951.1) and *O. clarkii* LRP13 (ALD16281.1), were aligned with DNAMAN software

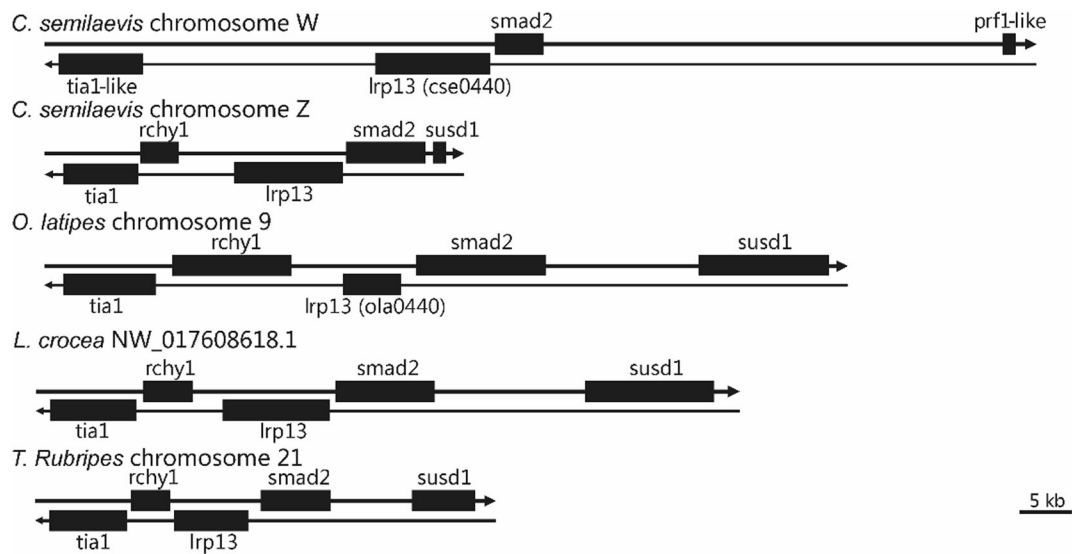


Fig. 3 The syntenic arrangements of LRP13 loci in four fish species. *C. semilaevis* chromosome W_12,122,334–12,216,605, *C. semilaevis* chromosome Z_2,019,894–2,071,435, *O. latipes* chromosome 9_25,175,067–25,248,101, *L. crocea* scaffold NW_017608618.1_2,267,866–2,329,685 and *T. rubripes* chromosome 21_5,289,031–5,329,390 were displayed with LRP13 and

other genes location relationships. *tia1*, TIA1 cytotoxic granule-associated RNA binding protein; *rchy1*, ring finger and CHY zinc finger domain containing 1; *smad2*, SMAD family member 2; *susd1*, sushi domain containing 1; *prf1-like*, perforin-1-like. Bar = 5 kb

mRNA was detected in the brain and the gonad, and the expression level in males was higher than that in females (Fig. 4a).

In situ hybridization further revealed that *cselrp13* mRNA was only detected to be expressed in the oocytes

and follicles of 150- and 311-day ovaries *C. semilaevis*. The expression level in follicles was higher than that in oocytes. In the testis, no obvious signals were observed (Fig. 5). And no signals were either detected at 44, 68 and 83 days of gonads.

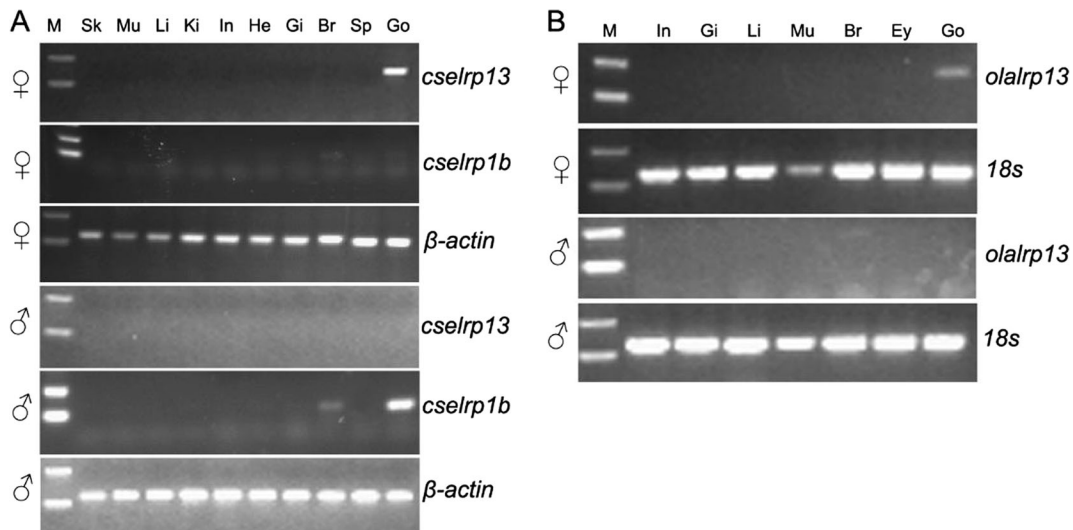
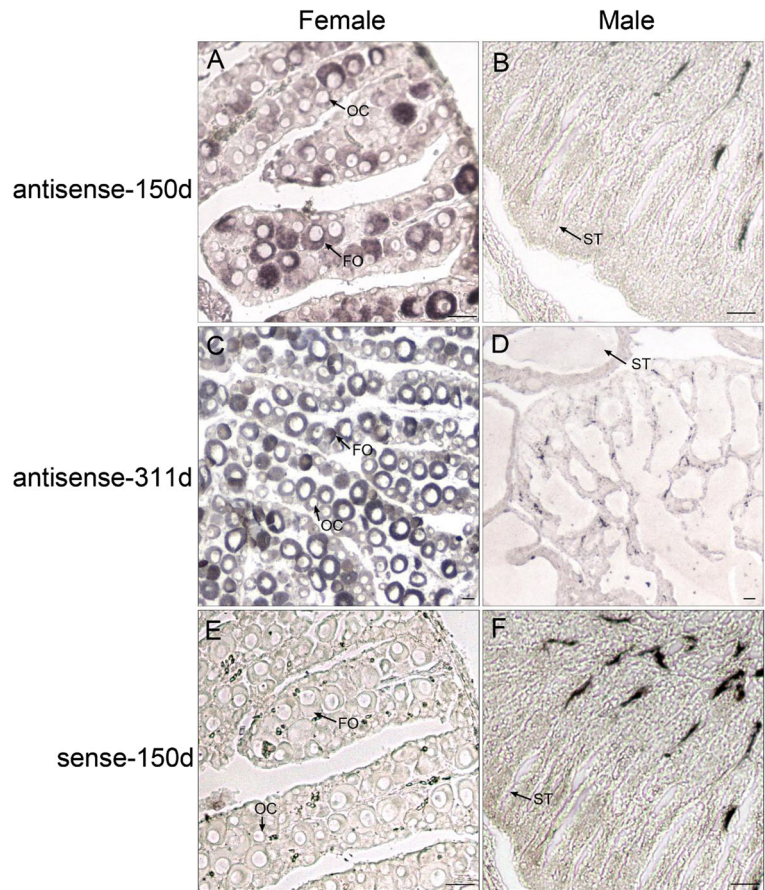


Fig. 4 The expression pattern of *cselrp13* and *cselrp1b* in *C. semilaevis* (a) and *olalrp13* in *O. latipes* (b). M DL2000 marker, Sk skin, Mu muscle, Li liver, Ki kidney, In intestine, He heart, Gi gill, Br brain, Sp spleen, Ey eye, Go gonad

Fig. 5 The in situ hybridization of *cselrp13* mRNA in *C. semilaevis*. **a, c** Female gonad slides at 150 and 311 days hybridized by *cselrp13* antisense probe, respectively. **b, d** Male gonad slides at 150 and 311 days hybridized by *cselrp13* antisense probe, respectively. **e, f** indicate the sense probe hybridization result in female and male gonad slides at 150 days. *OC* oocyte, *FO* follicle, *ST* spermatozoa. *Bar* = 50 μ m



Discussion

In the present study, we identified a female-specific gene in *C. semilaevis*, *cse0440*, which is initially annotated as LRP1B in NCBI database. The obvious discrepancy between *cse0440* and LRP1B in protein length and mRNA expression pattern has suggested that they are different genes. The LRP family is composed of at least 12 different gene members (<http://www.ncbi.nlm.nih.gov/homologene/?term=low+density+lipoprotein+receptor-related+protein>): LRP1–6, LRP8, LRP10–12, LRP1B and prolow-density lipoprotein receptor-related protein 1-like. The last paralogue (GeneBank HomoloGene 132,117), identified as LRP13, was predominantly expressed in the ovary of two teleost fishes, *M. saxatilis*, *M. americana* (Reading et al. 2014) and *O. clarkii* (Mushirobira et al. 2015). The similar expression pattern of *cse0440* and LRP13 implied their homology, which was later confirmed by phylogenetic analysis of 85 LRP proteins. Additional LRP13 orthologues were identified from other species, such as

M. musculus, *B. taurus*, *G. gallus*, *O. latipes*, *D. rerio*, *S. salar*, *T. rubripes* and *L. crocea*, which suggested its conserved distribution in mammals and fishes. Interestingly, *C. semilaevis* LRP2-like protein (XP_008333985.1), located on chromosome Z, was also identified as a homologue of LRP13. To avoid confusion, this protein was re-annotated as *cseLRP13-Z*, which shared 77.5% identity with *cseLRP13-W* in protein sequences. It is the first report of two LRP13 homologues existing in the genome of one species.

The identical syntenic arrangements of LRP13 on *C. semilaevis* chromosome W and chromosome Z, *O. latipes* chromosome 9, *L. crocea* NW_017608618.1 and *T. rubripes* chromosome 21 were observed to be similar to those of *D. rerio* and *O. niloticus* (Reading et al. 2014). The minor difference in *cseLRP13* on *C. semilaevis* chromosome W may be caused by incomplete assembly.

In summary, a lipoprotein receptor, LRP13, was identified and characterized from *C. semilaevis* and *O. latipes*. The exclusive expression in the ovary

revealed its specific roles in female reproduction. In *C. semilaevis* and *O. latipes*, whether *lrp13* played similar roles in binding vitellogenins with other fish LRP13 (Reading et al. 2014; Mushirobira et al. 2015) remains unclear and requires more investigation. It is expected that the advancement of micromanipulation in flatfish embryos may allow for the unveiling of the possible different functions of two *lrp13* genes in *C. semilaevis* reproduction and sex determination.

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Author contributions NW and SLC conceived and designed the experiments, while NW and QMH verified the sequences of cseLRP13 and olaLRP13 by PCR experiments. RQW conducted RT-PCR. YZ and FY participated in the experiment of in situ hybridization. NW and RQW analysed the data and wrote the paper. WTX provided valuable suggestions regarding paper organization and language fluency. All authors read and approved the final manuscript.

Compliance with ethical standards The collection and handling of the animals used in this study were approved by the Animal Care and Use Committee at the Chinese Academy of Fishery Sciences, and all the experimental procedures were performed in accordance with the guidelines for the Care and Use of Laboratory Animals at the Chinese Academy of Fishery Sciences.

Competing interest The authors declare that they have no competing interests.

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