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Identification of Sexually Dimorphic Genes in Pectoral Fin as Molecular Markers for Assessing the Sex of Japanese Silver Eels (*Anguilla japonica*)

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The Japanese eel (*Anguilla japonica*) is an important species in East Asian aquaculture. However, the production of seedlings for this purpose still depends on natural resources, as the commercial production of glass eels is not yet possible. Confusion about the sex of silver eels is one of the factors affecting the success rate of artificial maturation. This study sought to devise a harmless method to precisely assess the sex of silver eels. Partial pectoral fins were collected from females and males and the total RNA was extracted for transcriptomic analysis to identify sexually dimorphic genes as molecular markers for sex typing. An online database was constructed to integrate the annotations of transcripts and perform comparative transcriptome analysis. This analysis identified a total of 29 candidate sexually dimorphic genes. Ten were selected for a real-time quantitative polymerase chain reaction (RT-qPCR) to validate the transcriptomic data and evaluate their feasibility as markers. The transcriptomic analysis and RT-qPCR data implicated three potential markers (*LOC111853410*, *kera*, and *dcn*) in sex typing. The expression of *LOC111853410* was higher in females than in males. In contrast, the expression of *kera* and *dcn* was higher in males than in females. The ΔC_T values of three markers were analyzed to determine their inferred thresholds, which can be used to determine the sex of Japanese eels. The results suggested that if a silver eel had a pectoral fin with the pectoral fin having the ΔC_T of *LOC111853410* < 11.3, the ΔC_T of *kera* > 11.4, or the ΔC_T of *dcn* > 6.5 can be assessed it could be assessed as female. Males could be assessed by the ΔC_T of *LOC111853410* > 11.3, the ΔC_T of *kera* < 11.4, or the ΔC_T of *dcn* < 6.5 in their pectoral fins. The molecular functions of these markers and the biological significance of their differential expression require further exploration.

Key words: Comparative transcriptome analysis, Japanese eel, Pectoral fin, Sex typing, Sexually dimorphic genes.

BACKGROUND

The Japanese eel, *Anguilla japonica*, is a catadromous fish that spends most of its growth phase in freshwater habitats but spawns in the open ocean. Its spawning site is located in the west of the Mariana Ridge, as confirmed by the eggs and leptocephali collected in the water column (Tsukamoto 1992 2006). In recent years, overfishing, habitat destruction, and changes in the ocean environment have led to the

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drastic depletion of natural resources (Chen et al. 2014). Consequently, the Japanese eel has been listed on the International Union for Conservation of Nature Red List of Threatened Species as an endangered species since 2014 (Pike et al. 2020). Conserving the natural resources of the Japanese eel and ensuring a stable supply of seedlings for eel culture requires the development of techniques for artificial propagation.

The artificial propagation of Japanese eels has been studied for a long time (Okamura et al. 2014; Tanaka 2015). However, techniques for the mass production of glass eels have not yet been established. The reasons include unclear mechanisms of sex determination and differentiation, unrecognizable sexes for silver eels before artificial maturation, imperfect techniques of artificial maturation and an unstable supply of parent eels, unstable quality of fertilized eggs, unintelligible mechanisms of nutrient utilization and metabolism of eel larvae, and inappropriate feed and cultivation methods for eel larvae (Hsu et al. 2015; Okamura et al. 2014 2018 2020; Tanaka 2015; Tesch 2003; Yamada et al. 2019). An increasing number of studies are addressing these difficulties, with the goal of establishing techniques for the mass production of glass eels.

Karyological analyses of anguillid eels have suggested that they possess heteromorphic sex chromosomes (Park and Kang 1979; Park and Grimm 1981). However, the presence of heteromorphic sex chromosomes is not a determinant of sex determination and differentiation in eels (Wiberg 1983). Sex determination and differentiation of eels begins at the juvenile (yellow eel) stage (Colombo and Grandi 1996). Environmental factors, such as water temperature, population density, and body size of juvenile eels, have been suggested to override the genetic effects on sex determination and differentiation (Colombo et al. 1984; Colombo and Grandi 1996; Davey and Jellyman 2005; Oliveira and McCleave 2000; Tesch 2003). Higher water temperature and population density, and larger body size of juvenile eels may increase the proportion of male eels. Sex differentiation in eels has been associated with the expression of several sexually dimorphic genes/hormones in the brain and gonads (Geffroy et al. 2016; Inaba et al. 2021; Jeng et al. 2018). The expression of *amh*, *dmrt1*, *gsdf*, *sox9a*, and *vasa* significantly increased during testicular differentiation of eels. The expression of *cyp19a1*, *figla*, *foxl2a*, *foxn5*, *sox3*, *zar1*, and *zp3* was high during the ovarian differentiation of eels. Treatment with estrogens had a feminization effect on fish, including eels (Chiba et al. 1993; Colombo and Grandi 1995; Guiguen et al. 2010). Observations have indicated a higher proportion of females in wild eel populations compared to that in farmed eels due to the low population density. However, the detailed mechanisms controlling the expressions of sexually dimorphic genes remain unclear (Chu et al. 2006; Han and Tzeng 2006).

Eel biologists have speculated that appearance characteristics, such as asymptotic length, eye size, and shape of the pectoral fin, vary between sexes (Tesch 2003). However, even after long-term observation, we found it difficult to precisely identify the sex of Japanese silver eels that completed sex differentiation based on their appearance characteristics, which may be affected by genetics, age, and environment. In a previous study on the reproduction of European eels, du Colombier et al. (2015) introduced ultrasonography as a noninvasive tool to assess the sex and maturity of European silver eels. The accuracy of this technique was insufficient. Additionally, a recent report mentioned that performing anesthesia surgery to sample a piece of gonadal tissue for histological analysis can determine the sex of Japanese silver eels, but it involves risks of causing injury, inflammation, and even death to the eels (Huang et al. 2020). Therefore, the development of a new technique for assessing the sex of silver eels with no harm and high accuracy at the same time is essential and would also be helpful for the artificial propagation of eels. Because of the discrepancy in the procedures of artificial maturation for females and males, such as the required time and hormone combination, knowing the sex of silver eels beforehand would improve the efficiency, success rate, and costs of artificial maturation (Okamura et al. 2014; Tanaka 2015). Subsequently, controlling the sex ratio of each batch of parent eels would assist researchers in arranging the start point of artificial maturation for each eel and the mating time among parent eels, which would increase the success rate of fertilization and the number of fertilized eggs to obtain a large number of leptocephali for cultivation (Okamura et al. 2020).

This study aimed to devise a method for precisely and harmlessly assessing the sex of silver eels before artificial maturation. In addition to the brain and gonads, sexually dimorphic genes may also exist in other body tissues of the eels. Thus, a piece of the pectoral fins of two female and two male silver eels was collected for RNA-sequencing (RNA-seq). A comparative transcriptome analysis identified candidate sexually dimorphic genes, which may serve as markers for sex typing in Japanese silver eels. RT-qPCR experiments validated the transcriptomic data and assessed the feasibility of these genes as molecular markers. If there are available molecular markers for sex typing, it will improve the artificial maturation of Japanese eels. Moreover, introducing a similar research strategy to discover the molecular markers for assessing the

gonadal maturity of Japanese eels during artificial maturation can also be possible.

MATERIALS AND METHODS

Sample collection

Ten Japanese silver eels comprising five females (body weight 675–900 g) and five males (body weight 460–575 g) were purchased from a local eel farmer in Pingtung in southern Taiwan. On the basis of our previous study (Han et al. 2003), silver eels (adult stage) could be differentiated from yellow ones (juvenile stage) by their morphometric characteristics, such as skin coloration, total length, ocular index (OI), and fin-index (FI). The eels were acclimated to seawater in a two-ton tank at the laboratory for approximately two weeks for subsequent artificial maturation. Samples of pectoral fins were collected from all ten anesthetized silver eels before the artificial maturation. The biopsy samples were immediately stored in a -80°C freezer. Biopsy samples of two females and two males were randomly selected for RNA-seq, and the remaining biopsies were reserved for subsequent RT-qPCR analysis. Additional pectoral fin tissue biopsied from other silver eels was sampled for RT-qPCR analysis. Two females (body weights of 658 g and 705 g) and two males (body weights of 489 g and 618 g) were purchased from a commercial eel dealer in Tainan, southern Taiwan. Two female eels (body weights of 420 g and 560 g) were reared from elvers and fed an E2-containing diet (Chiba et al. 1993; Colombo and Grandi 1995; Guiguen et al. 2010) at the culture station of National Taiwan University for 24 months. Pre-sampling handling of silver eels and post-sampling storage of biopsies was performed as described above. Detailed information on all the silver eels is shown in table S1.

In addition, ten cultured eels at the juvenile stage were bought from an eel farmer in Pingtung to sample the pectoral fins for RT-qPCR analysis. All of them were male, and no female juvenile eel was obtained in this study. These juvenile eels were reared in a two-ton tank with freshwater for three days after arriving at the laboratory to ease their stress. Subsequently, they were sacrificed for the sampling and measurement of morphometric characteristics. Detailed information on these juvenile eels is displayed in table S2. All procedures and investigations were approved by the Institutional Animal Care and Use Committee of the National Taiwan University (NTU107-EL-00059) and executed in accordance with standard guidelines.

RNA extraction, library construction, and sequencing

Total RNA from the entire mass of the biopsied tissue was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified RNA was quantified using an ND-1000 spectrophotometer (Nanodrop, Wilmington, DE, USA) and characterized using a Bioanalyzer 2100 with an RNA 6000 Labchip kit (Agilent Technologies, Santa Clara, CA, USA). After the Bioanalyzer 2100 analysis, the RNA integrity numbers of all RNA samples prepared in this study exceeded 7.0. Sequencing libraries were constructed using the TruSeq RNA Sample Prep Kits v2 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. They were subsequently sequenced using the Illumina HiSeq-2500 platform.

Processing of sequence data, gene prediction, and gene annotation

Four datasets included two female pectoral fins (FPF1: ERS13509836 and FPF2: ERS13509837) and two male pectoral fins (MPF1: ERS13509838 and MPF2: ERS13509839), which have been deposited in European Nucleotide Archive (ENA) of European Bioinformatics Institute (EBI). Raw RNA-seq data were filtered using the TrimGalore program (Babraham Bioinformatics, Cambridge, UK) to discard adaptors and low-quality reads ($Q < 13$). Low-complexity reads (repeat sequences) were removed using the prinSeq program. Finally, the general read properties were generated using the FastQC program (Babraham Bioinformatics).

The MAKER2 pipeline (https://www.yandell-lab.org/software/maker.html) was used to predict genes and generate the gene transfer format (GTF). The input datasets included the four RNA-seq datasets obtained in this study, previously published RNA-seq data (Hsu et al. 2015), PacBio Iso-Seq data, newly established Japanese eel genome (unpublished data), available protein sequences of *A. japonica*, all teleosts from the UniProt database, and the pre-existing zebrafish gene model. The new version of the Japanese eel genome established by our team had 1.06 Gb with 4,861 scaffolds and N50 over 4.6 Mb. By taking 448 scaffolds with lengths larger than 100 Kb, these scaffolds can cover 98% of the Japanese eel genome. The predicted open reading frames were annotated with homologs in the updated NR database (April 2019) using GhostX (Suzuki et al. 2014). Protein sequence features, including signal peptides, transmembrane domains, and domains described in the Pfam database, were detected using SignalP (Petersen et al. 2011), TMHMM

(Krogh et al. 2001), and HMMER (Mistry et al. 2013), respectively. Gene ontology annotation was performed for genes with detectable Pfam domains according to Pfam2GO. Protein-coding genes were mapped to the canonical Kyoto Encyclopedia of Genes and Genomes (KEGG) database pathway using the KEGG Automatic Annotation Server (Moriya et al. 2007). The parameters were set to map to only related eukaryotic databases in the single-directional best-hit mode.

Construction of database framework and estimation of gene expression

An online database (MOLAS) for the Japanese eel was constructed using the LAMP system architecture (Ubuntu 14.04, Apache 2.04, PHP 5.1 and MySQL 8.0) with the Bootstrap 3 CSS framework (http://getbootstrap.com/), jQuery1.11.1, and jQuery Validation v1.17 to provide an intuitive user experience. The entire system runs on a virtual machine on the cloud infrastructure of the Institute of Information Science, Academia Sinica, Taiwan. The analytical process was implemented using scripts written in R (3.4.2).

According to the assembly with GTF, the gene expression profiles can be estimated using raw reads generated from RNA-seq via an intuitive graphical interface in Docexpress (https://hub.docker.com/r/lsbnb/docexpress_fastqc) with a built-in process of Hisat2 -> StringTie -> Ballgown. Finally, the gene expression profiles in fragments per kilobase of exon per million fragments mapped (FPKM) values were submitted to MOLAS implemented by our team.

Comparative analysis between female and male pectoral fin libraries for gene expression profiles

In this study, the "Pairwise Comparison" function on the online database was first used to compare the datasets of the female and male pectoral fins to find the differentially expressed genes (highly expressed in females but minimally in males, and vice versa). Two female pectoral fin datasets (FPF1 and FPF2) and two male pectoral fin datasets (MPF1 and MPF2) were individually pooled for this analysis. According to previous studies on sexually dimorphic genes in Japanese eels (Geffroy et al. 2016; Inaba et al. 2021; Jeng et al. 2018), the expression levels of most sexually dimorphic genes were significantly different between female and male eels, which was over five fold-change. Therefore, screening parameters were set such that the expression levels (FPKM value) of transcripts were ≥ 0, and the differences in expression levels of transcripts were at least six-fold between the two pools. Then, the "Clustering" function was used to categorize the expression patterns of the transcripts identified from the above step and remove the transcripts that were highly expressed in only one dataset of female or male pool and that were minimally expressed in four datasets of female and male pool. Thereafter, the transcripts with higher expression in females and males were separately filtered out to identify candidate genes for assessing the sex of Japanese eel.

RT-qPCR

The specific primer pairs of candidate genes for RT-qPCR were designed using the Primer 3 software and listed in table 1. The specificity of each primer pair was verified using PCR and electrophoresis, and amplicons were validated using DNA sequencing. In addition, the qPCR efficiency of each primer pair was tested by serially diluting cDNA samples from eel pectoral fin, as listed in table 1. The cDNA of each specimen was reverse transcribed from 1 μg of total RNA with four repeats using iScript cDNA Synthesis kits (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. RT-qPCR was performed using a MyiQ real-time PCR system (Bio-Rad) with a 25 μL mixture containing 12.5 μL of 2× SYBR Green Supermix (Bionova, Fremont, CA, USA), 1 μL of each primer, 1 μL of cDNA template (10× dilution), and 9.5 μL of distilled deionized water. The running conditions followed were pre-incubation at 95°C for 10 min and 40 amplification cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 45 s. Acidic ribosomal phosphoprotein P0 (*arp*), which is consistently expressed at the same level in eels, was used as a housekeeping gene (Sudo et al. 2013). RT-qPCR data were analyzed in two steps. First, *arp* was used for normalization by subtracting the cycle threshold (C_T) value from the C_T value of the target gene in every sample to get the ΔC_T. Second, in each gene category, two female samples (F1 and F2) were used as the control for normalization by subtracting the average ΔC_T value of them from the ΔC_T value of each sample to get the $\Delta\Delta C_T$. The relative mRNA expression levels were calculated as $2^{-(\Delta\Delta C_T)}$ and were presented as the Vertical Box Plot with two groups (S-Female and S-Male) and the Vertical Bar Chart with all the samples in each gene category. Besides, in another analysis for defining the criteria for sex typing of silver eels, the ΔC_T (normalization with *arp*) values of candidate sexually dimorphic genes were compared between the S-Female and S-Male groups. Then, the threshold of ΔC_T of each candidate gene was inferred by summing the lowest ΔC_T of the group having higher ΔC_T values with the highest ΔC_T of the other group and taking the average. The ΔC_T values of every candidate gene in the S-Female and

S-Male groups were presented as the Vertical Point Plot.

Statistical analyses

For the statistical test on the differentially expressed transcripts that have six fold-change between the female pool (FPF) and male pool (MPF), we applied the R package, *limma*, to perform linear modeling on the expression levels (FPKM) and used the empirical Bayes method to moderate gene-wise variability, generating *P* values (Ritchie et al. 2015). The *P* values were adjusted by the Benjamini-Hochberg (BH) method (Benjamini and Hochberg 1995). The expression values of ten genes in all samples from RT-qPCR and the ΔC_T values of three candidate sexually dimorphic genes were statistically analyzed using the SAS version 9.1 statistical software (SAS Institute Inc., Cary, NC, USA). The expression values of ten genes in all samples from RT-qPCR were analyzed using one-way ANOVA followed by a Student-Newman-Keuls multiple comparison test, with $P < 0.05$ indicating a significant difference. Student's *t*-test evaluated the differences in the expression values of ten genes between the S-Female and S-Male groups in the Vertical Box Plot and the differences in the ΔC_T values of three candidate sexually dimorphic genes between the S-Female and S-Male groups in Vertical Point Plot, with $P < 0.05$ indicating a significant difference.

RESULTS

Next-generation sequencing statistics and establishment of online database

An average of approximately 11.54 million raw reads, excluding those for ribosomal RNA and mitochondrial RNA, of nearly 150 base pairs (bp) in length were obtained for each library (see Table S3). The raw data of each library were cleaned up and mapped to the Japanese eel genome (see Table S3), followed by gene prediction, functional annotation, and estimation of expression levels of transcripts. A gene model containing 27,350 Japanese eel transcripts was constructed. The transcripts were blasted against the NR database, and a total of 27,336 best hits were identified. In addition, the annotated information and expression levels of all transcripts were obtained after a series of functional annotations and calculations and integrated into the MOLAS (https://molas.iis.sinica.edu.tw/2022_jpeel/login.php) database established by our research team (Fig. 1).

Table 1. Specific primer pairs used for real-time quantitative PCR analyses (F, forward strand; R, reverse strand)

Transcript ID	Hit_seq name	Gene name on NCBI	Primer sequences	Amplicon size (bp)	qPCR efficiency
AJA4564.p1	septin-2-like isoform X2	*LOC108249696*	F: 5'- AACAGGAACAGGGAAGTCTG -3' R: 5'- TCGGAGCTGATCTTGTCTTC -3'	84	99.9%
AJA12577.p1	heat shock protein 30-like	*LOC110515957*	F: 5'- TGAGAAGACCCTGCAGTTCC -3' R: 5'- TGCTGGTGATTCTGTGCTTC -3'	69	99.8%
AJA24273.p1	early growth response protein 3	*egr3*	F: 5'- TCCTGGGAAGCGGGAGAG -3' R: 5'- CCAGAGAAGTACCACGGTCG -3'	137	99.5%
AJA16680.p1	CLOCK-interacting pacemaker	*cipc*	F: 5'- GAGTCAGAGAGGGACTCTGG -3' R: 5'- GTAGGACCCCGTCATCACC -3'	136	99.6%
AJA21056.p1	mucin-19-like	*LOC111853410*	F: 5'- GCAACTGTGAAGGCAACAAG -3' R: 5'- ACTGTTACAAGGTACCACGC -3'	128	99.6%
AJA20026.p1	src substrate cortactin	*cttn*	F: 5'- ATGTGTACCAGTCGGAGCC -3' R: 5'- CACTGCCGAACCTCCTCTAC -3'	229	98.8%
AJA14877.p1	keratocan isoform X1	*kera*	F: 5'- TCCCAATGCTCGTACTTTCG -3' R: 5'- TGCTTCAGGTTCCTGTTGTC -3'	214	99.1%
AJA20844.p1	decorin precursor-like	*dcn*	F: 5'- ACAACAAGCTCCAGACCATC -3' R: 5'- AGGTGCCTCAGTTTGGAGTA -3'	139	99.5%
AJA7588.p1	cbp/p300-interacting transactivator 1	*cited1*	F: 5'- GCATCATCGATTCGGACCC -3' R: 5'- CTCAGCAATGGTCAAACGGA -3'	188	99.4%
AJA1691.p1	amine sulfotransferase-like	*LOC104575574*	F: 5'- GTTCTTGGAGAGAGGCATGG -3' R: 5'- TCGCTGATCTTCACAACGAC -3'	159	99.4%
-		*arp*	F: 5'- GTGCCAGCTCAGAACACTG -3' R: 5'- ACATCGCTCAAGACTTCAATGG -3'	107	99.8%

Comparative analysis between female and male pectoral fin libraries for gene expression profiles

The "Pairwise Comparison" and "Clustering" functions on the database were used to compare the libraries of pectoral fins of female and male eels to discover differentially expressed transcripts. After parameter setting and operation of the platform, a total of 15 transcripts displayed high expression levels in the pectoral fins of female eels but low or almost no expression in the pectoral fins of male eels. The detailed

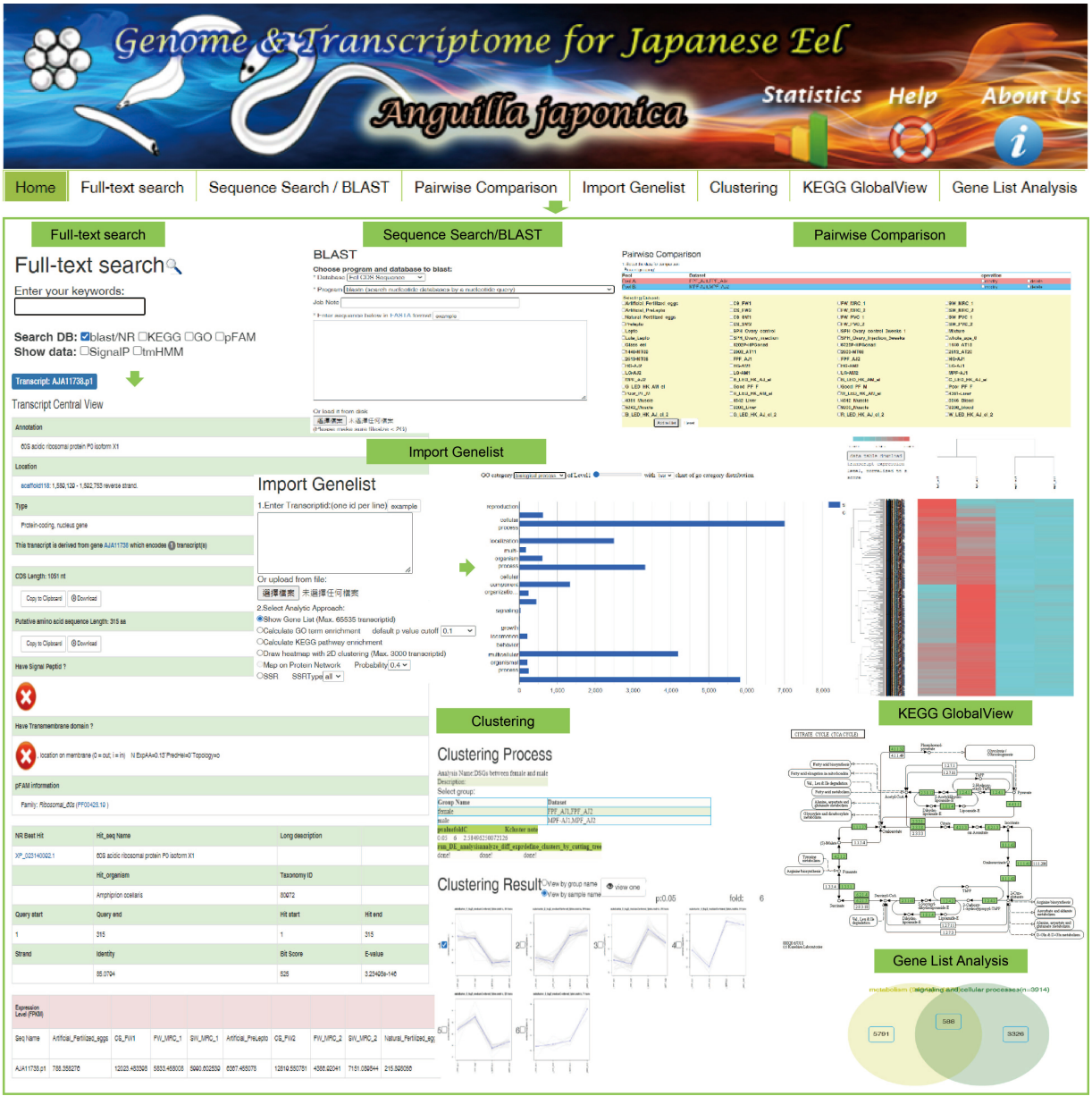

Fig. 1. Scheme of the MOLAS database for the Japanese eel. Annotated information of the assembled transcripts, including DNA sequence, amino acids sequence, open reading frames, signal peptides, transmembrane domains, gene ontology, hit KEGG pathway, and FPKM values, can be searched by the names of genes or the transcript ID in the Full-text search. A sequence of DNA can also be used to find the transcript with high similarity through the Sequence Search/BLAST. Moreover, the Pairwise Comparison can compare the differences in expression levels of the transcripts between two different libraries or two library groups, and then the differentially expressed genes can be summarized to a gene list. Furthermore, the Import Genelist can analyze the protein function, gene ontology enrichment, heatmap of expression, and hit terms on a KEGG pathway for a gene list. Additionally, the Clustering can be used to categorize the expressional patterns of transcripts between two different libraries or two library groups. Finally, the KEGG GlobalView and Gene List Analysis can see the hit terms of transcripts on the map of KEGG pathway and compare the different gene lists by Venn diagrams, respectively.

annotations of these transcripts are listed in table 2. Fourteen other transcripts displayed higher expression levels in the pectoral fins of male eels but low or almost no expression levels in the pectoral fins of females. The detailed annotations of the 14 transcripts are presented in table 3. These twenty-nine differentially expressed transcripts all pass the statistical test ($P < 0.05$) which implies that they indeed have significant differences in expression levels between the female pool and male pool. The adjusted *P* values are shown in table 2 and table 3. The sequence information of these twenty-nine transcripts is presented in table S4. These differentially expressed transcripts may be sexually dimorphic genes in the pectoral fin of the Japanese eel. Subsequently, ten of these transcripts were selected for RT-qPCR to validate the transcriptomic data and assess their feasibility as molecular markers. The expression levels of six candidates in the pectoral fins of female eels were higher than those in the pectoral fins of males. On the other hand, the expression levels of four candidates were higher in the pectoral fins of male eels than in the pectoral fins of females.

RT-qPCR of pectoral fin tissues from female and male silver eels

Ten candidate genes obtained from pectoral fin tissues of seven female (F1–F7) and five male (M1–

Table 2. Information of transcripts whose expression in the libraries of pectoral fins of female silver eels was six-fold higher than that of male silver eels

Transcript ID	CDS length	NR best hit	Best hit_E value	Hit_seq name
AJA12763.p1	453 nt	KKF25601.1	4.689e-15	Protein NLRC3
AJA1330.p1	1221 nt	XP_018604518.1	1.651e-141	galactosylceramide sulfotransferase-like
AJA14095.p1	791 nt	XP_018924236.1	1.715e-60	protein fosB-like isoform X4
AJA15563.p1	1080 nt	XP_018594957.1	1.641e-155	fatty acid 2-hydroxylase
AJA16680.p1	462 nt	XP_023684314.1	5.780e-37	CLOCK-interacting pacemaker
AJA18689.p1	1398 nt	XP_020340178.1	1.494e-128	otopetrin-1
AJA20026.p1	345 nt	XP_021180512.1	1.277e-20	src substrate cortactin
AJA21056.p1	508 nt	XP_023686008.1	1.384e-35	mucin-19-like
AJA24273.p1	2355 nt	XP_018599663.1	4.190e-210	early growth response protein 3
AJA25664.p1	489 nt	XP_023663962.1	2.182e-52	class I histocompatibility antigen, F10 alpha chain-like
AJA12577.p1	2210 nt	XP_021450334.1	1.637e-64	heat shock protein 30-like
AJA27270.p1	267 nt	KPP63944.1	9.285e-27	Annexin A11-like, partial
AJA4564.p1	396 nt	XP_017294726.1	2.513e-08	septin-2-like isoform X2
AJA4608.p1	1263 nt	XP_017579547.1	1.058e-106	tyrosine-protein kinase fyna
AJA737.p1	1071 nt	OWK00640.1	1.469e-23	MBNL2, partial

Transcript ID	Hit_organism	Involved function	Adjusted *P* value from *limma* R package
AJA12763.p1	*Larimichthys crocea*	Ribonuclease inhibitor	3.76e-05
AJA1330.p1	*Scleropages formosus*	Galactosylceramide sulfotransferase activity	5.25e-05
AJA14095.p1	*Cyprinus carpio*	DNA-binding transcription factor	7.70e-06
AJA15563.p1	*Scleropages formosus*	Lipid biosynthetic process	2.64e-05
AJA16680.p1	*Paramormyrops kingsleyae*	Negative regulation of transcription	0.000274
AJA18689.p1	*Oncorhynchus kisutch*	Formation of otoconia and proton channels	0.000258
AJA20026.p1	*Fundulus heteroclitus*	Actin filament polymerization	7.95e-06
AJA21056.p1	*Paramormyrops kingsleyae*	Formation of mucin protein	0.000215
AJA24273.p1	*Scleropages formosus*	DNA binding	2.87e-05
AJA25664.p1	*Paramormyrops kingsleyae*	MHC class I antigen	1.11e-06
AJA12577.p1	*Oncorhynchus mykiss*	Structural constituent of ribosome and phospholipid binding	2.57e-06
AJA27270.p1	*Scleropages formosus*	Calcium-dependent phospholipid binding	0.000653
AJA4564.p1	*Kryptolebias marmoratus*	Transmembrane transporter activity, GTP-binding activity, and innate immunity	0.002781
AJA4608.p1	*Pygocentrus nattereri*	Tyrosine-protein kinase activity	2.56e-06
AJA737.p1	*Cervus elaphus hippelaphus*	RNA splicing protein	4.44e-05

M5) silver eels were subjected to RT-qPCR. The results of RT-qPCR displayed that the expression levels of *cipc*, *LOC111853410*, and *cttn* in the female silver eel group were statistically significantly higher than those in the male silver eel group (Fig. 2D–F). However, there were no significant differences in the expression of *cipc* or *cttn* among some female and male silver eel samples (Fig. S1D and Fig. S1F). In addition, the expression levels of *LOC108249696*, *LOC110515957*, and *egr3* had no significant differences between the female and male silver eel groups, as shown in figure 2A–C. On the other hand, the RT-qPCR showed that the expression levels of *kera*, *dcn*, *cited1*, and *LOC104575574* were significantly higher in the male silver eel group than in the female silver eel group (Fig. 3). However, there were no significant differences in the expression of *cited1* or *LOC104575574* among some female and male silver eel samples (Fig. S2C and Fig. S2D). Detailed expression levels of ten candidate genes in the pectoral fin tissues from all silver eel samples are displayed in figures S1 and S2. These RT-qPCR results indicated that the expression of *LOC111853410*, *kera*, and *dcn* is consistent with the transcriptomic data and suggested that only *LOC111853410*, *kera*, and *dcn* may be potential sexually dimorphic genes used for sex typing of silver eels.

Possible criteria for sex typing of silver eels

The best potential candidate sexually dimorphic genes, *LOC111853410*, *kera*, and *dcn*, were selected after analyses of transcriptomic data and RT-qPCR. The ΔC_T values of these three candidate genes from the pectoral fins of silver eels were individually analyzed to

Table 3. Information of transcripts whose expression in the libraries of pectoral fins of male silver eels was six-fold higher than that of female silver eels

Transcript ID	CDS length	NR best hit	Best hit_E-value	Hit_seq name
AJA11404.p1	495 nt	XP_016828489.1	5.355e-35	peptidyl-prolyl cis-trans isomerase FKBP11 isoform X5
AJA13052.p1	822 nt	BAP63925.1	1.385e-149	type I collagen proa2 chain
AJA14877.p1	1933 nt	XP_003445032.2	2.642e-132	keratocan isoform X1
AJA1532.p1	420 nt	XP_012692196.1	2.741e-63	collagen alpha-1(X) chain-like
AJA1691.p1	702 nt	XP_010221213.1	3.972e-55	amine sulfotransferase-like
AJA20844.p1	1038 nt	KPP76630.1	1.960e-134	decorin precursor-like
AJA21947.p1	707 nt	XP_006629547.2	4.719e-125	secreted frizzled-related protein 2
AJA22857.p1	582 nt	XP_023655487.1	5.314e-81	mimecan
AJA26246.p1	297 nt	XP_015457250.1	3.073e-43	SH3 domain-binding glutamic acid-rich-like protein
AJA2914.p1	327 nt	ACN78878.1	4.287e-53	glutathione peroxidase 1
AJA461.p1	372 nt	XP_012680047.1	8.861e-22	2-oxoisovalerate dehydrogenase beta subunit, mitochondrial
AJA5036.p1	360 nt	ELW64932.1	3.881e-41	DCN1-like protein 5
AJA7588.p1	921 nt	XP_007242076.1	2.743e-54	cbp/p300-interacting transactivator 1
AJA8438.p1	416 nt	XP_020796713.1	9.756e-13	Cytochrome c1, heme protein, mitochondrial

Transcript ID	Hit_organism	Involved function	Adjusted *P* value from *limma* R package
AJA11404.p1	*Cricetulus griseus*	Prolyl isomerase activity	0.000213
AJA13052.p1	*Anguilla japonica*	Formation of type I collagen	4.52e-08
AJA14877.p1	*Oreochromis niloticus*	Formation of keratocan	2.26e-06
AJA1532.p1	*Clupea harengus*	Formation of type X collagen	4.20e-07
AJA1691.p1	*Tinamus guttatus*	Amine sulfotransferase activity	0.000215
AJA20844.p1	*Scleropages formosus*	Formation of an extracellular matrix protein	5.01e-08
AJA21947.p1	*Lepisosteus oculatus*	Inhibition of osteoblast differentiation	2.33e-05
AJA22857.p1	*Paramormyrops kingsleyae*	As a osteoglycin involved in the regulation of energy homeostasis	3.25e-06
AJA26246.p1	*Astyanax mexicanus*	Electron transfer activity	0.000792
AJA2914.p1	*Anguilla japonica*	Response to oxidative stress and glutathione peroxidase activity	2.02e-06
AJA461.p1	*Clupea harengus*	Formation of beta subunit of 2-oxoisovalerate dehydrogenase	0.000747
AJA5036.p1	*Tupaia chinensis*	Formation of an extracellular matrix protein	0.000464
AJA7588.p1	*Astyanax mexicanus*	Regulation of transcription, DNA-templated	1.00e-05
AJA8438.p1	*Boleophthalmus pectinirostris*	Electron transfer activity	8.31e-05

establish the criteria for sex typing. The Vertical Point Plot of every candidate gene shows that the ΔC_T values of the S-Female and S-Male groups are clearly separated (Fig. 4). Moreover, there was a significant difference ($P < 0.05$) in the ΔC_T values between the S-Female and S-Male groups for every candidate gene. For the gene of *LOC111853410*, the lowest ΔC_T of the S-Male group and the highest ΔC_T of the S-Female group were summed and averaged to infer the threshold of ΔC_T for sex typing (Fig. 4A). In addition, for the genes of *kera* and *dcn*, the lowest ΔC_T of the S-Female group and the highest ΔC_T of the S-Male group were summed and averaged to infer the threshold of ΔC_T (Fig. 4B and 4C). The inferred thresholds of ΔC_T of *LOC111853410*, *kera*, and *dcn* were 11.3, 11.4, and 6.5, respectively. The analytic results indicated that the pectoral fins of female silver eels had the ΔC_T of *LOC111853410* < 11.3, the ΔC_T of *kera* > 11.4, and the ΔC_T of *dcn* > 6.5.

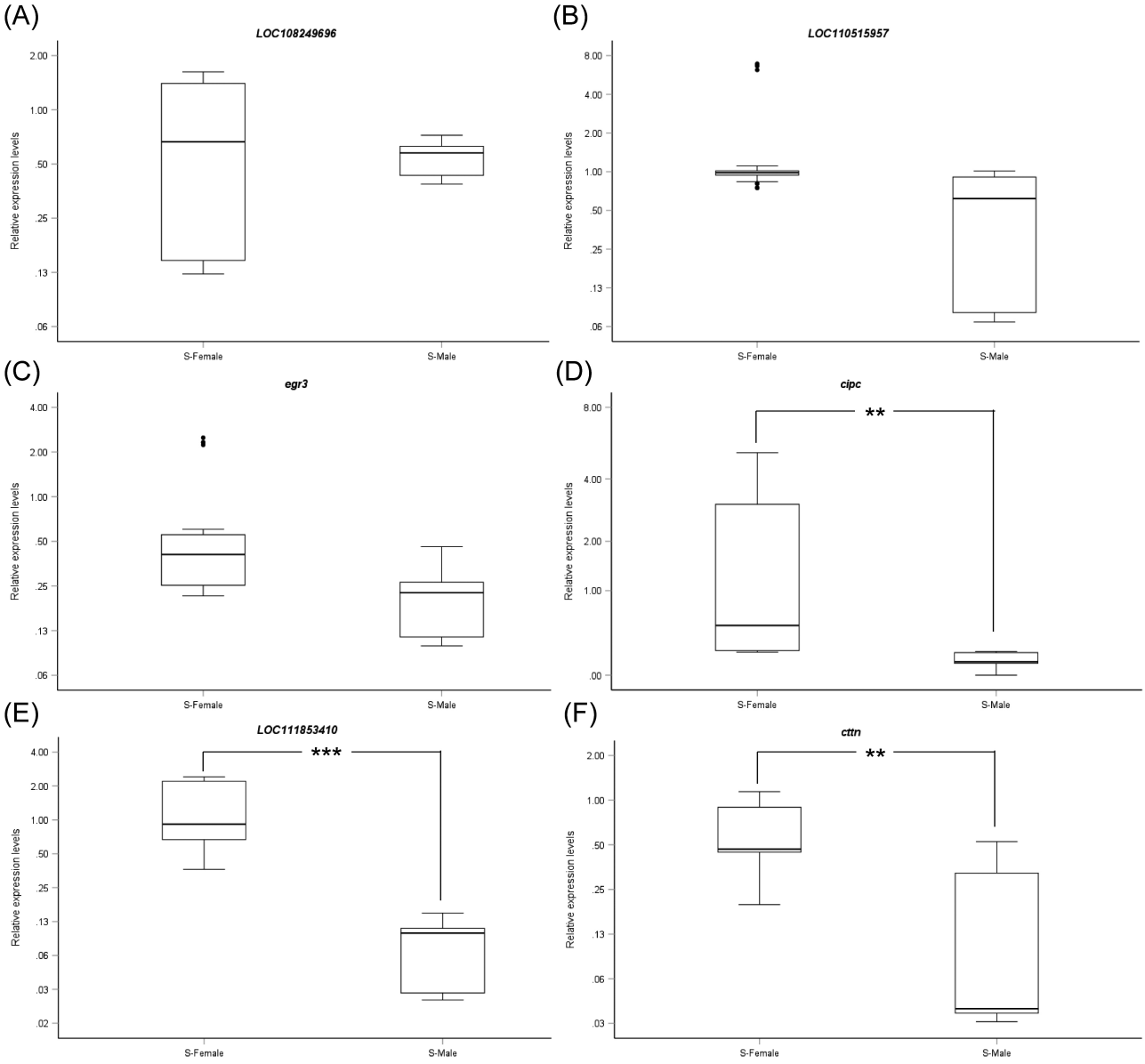

Fig. 2. Comparisons of relative expression levels of six genes possibly higher expressed in the pectoral fins of female silver eels between the "S-Female" (n = 7, four repeats) and "S-Male" (n = 5, four repeats) groups. (A) *LOC108249696*, (B) *LOC110515957*, (C) *egr3*, (D) *cipc*, (E) *LOC111853410*, (F) *cttn*. The *arp* was a housekeeping gene, and two female samples (F1 and F2) were used as the internal control for calculating relative expression levels. The y-axis was in $\log_2$ scale. The "S-Female" meant the female silver eels, and the "S-Male" meant the male silver eels. The solid black line represented the median value, and the solid black circle represented the outliers. Asterisks indicated significant differences (**$P < 0.01$, ***$P < 0.001$) in relative expression levels between two groups.

Conversely, the pectoral fins of male silver eels had the ΔC_T of *LOC111853410* > 11.3, the ΔC_T of *kera* < 11.4, and the ΔC_T of *dcn* < 6.5.

Testing the feasibility of candidate genes applied to sex typing for juvenile eels

In order to see whether the above ten candidate genes could be applied to assess the sex of juvenile eels (yellow eels), the ten genes were subject to RT-qPCR. The RT-qPCR results indicated that relative expression levels of these genes in the pectoral fins of juvenile male eels were similar to those in the pectoral fins of adult male eels (silver eels), as shown in figures S1 and S2, and suggested that *LOC111853410*, *kera*, and *dcn* may also be the markers for the sex typing of juvenile eels. Therefore, the ΔC_T values of three markers from the pectoral fins of juvenile male eels were further examined and compared with those from the pectoral fins of adult male eels (Fig. S3). The results showed that the inferred thresholds of ΔC_T of *LOC111853410* (11.3), *kera* (11.4), and *dcn* (6.5) from adult eels can also be applicable to juvenile eels (yellow eels) for sex typing. All the pectoral fins of juvenile male eels had the ΔC_T of *LOC111853410* > 11.3, the ΔC_T of *kera* < 11.4, and the ΔC_T of *dcn* < 6.5 (Fig. S3).

DISCUSSION

The Japanese eel is one of the most mysterious species worldwide because of its long and complex life history. Researchers have studied many physiological regulatory mechanisms in the Japanese eel, including larval development and metamorphosis, habitat use, migration between rivers and seas, nutrient metabolism, sex differentiation, natural gonad maturation, and reproduction (Chen et al. 2018; Hamidoghli et al. 2019; Hsu et al. 2015 2019; Inaba et al. 2021; Lee and Lou 2019; Okamura et al. 2018 2020). However, these physiological mechanisms are still not clearly understood, which has hindered the commercial-

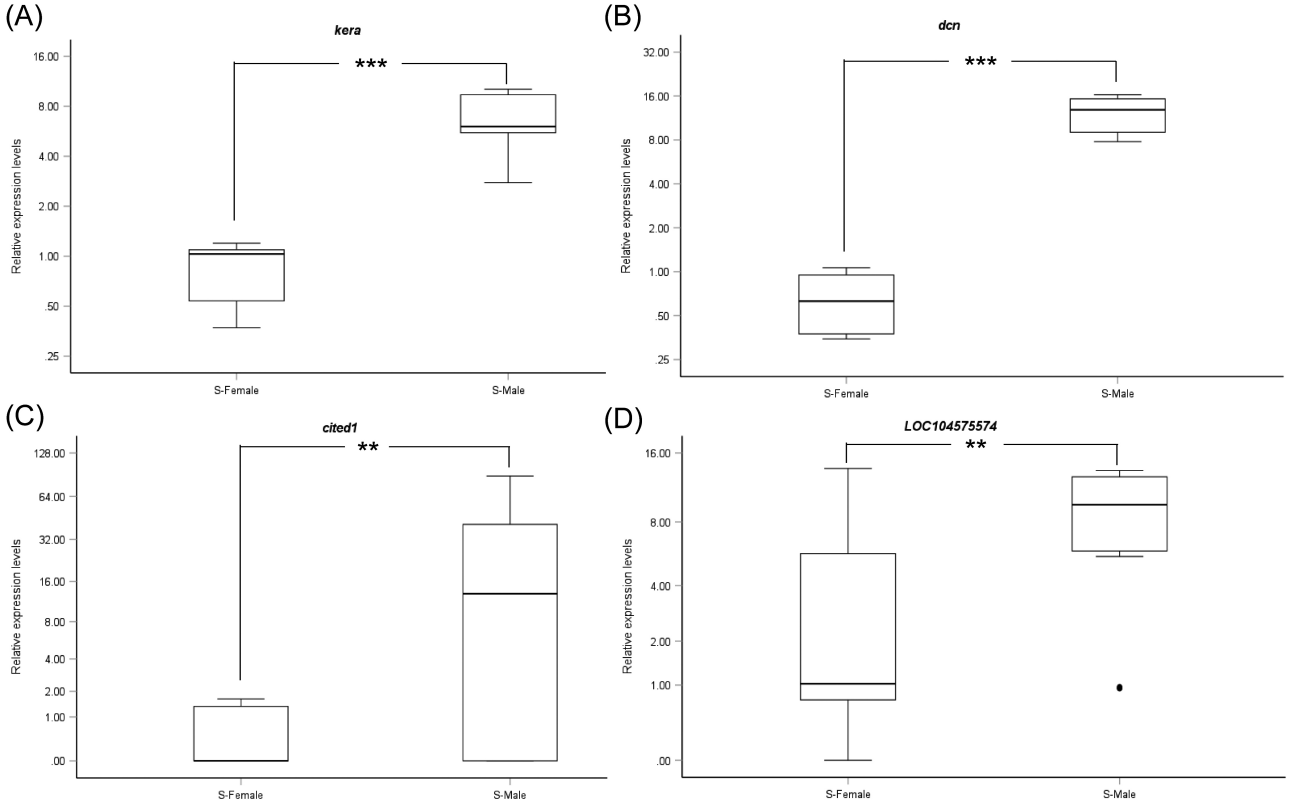

Fig. 3. Comparisons of relative expression levels of four genes possibly higher expressed in the pectoral fins of male silver eels between the "S-Female" (n = 7, four repeats) and "S-Male" (n = 5, four repeats) groups. (A) *kera*, (B) *dcn*, (C) *cited1*, (D) *LOC104575574*. The *arp* was a housekeeping gene, and two female samples (F1 and F2) were used as the internal control for calculating relative expression levels. The y-axis was in $\log_2$ scale. The "S-Female" meant the female silver eels, and the "S-Male" meant the male silver eels. The solid black line represented the median value, and the solid black circle represented the outliers. Asterisks indicated significant differences (**P < 0.01, ***P < 0.001) in relative expression levels between two groups.

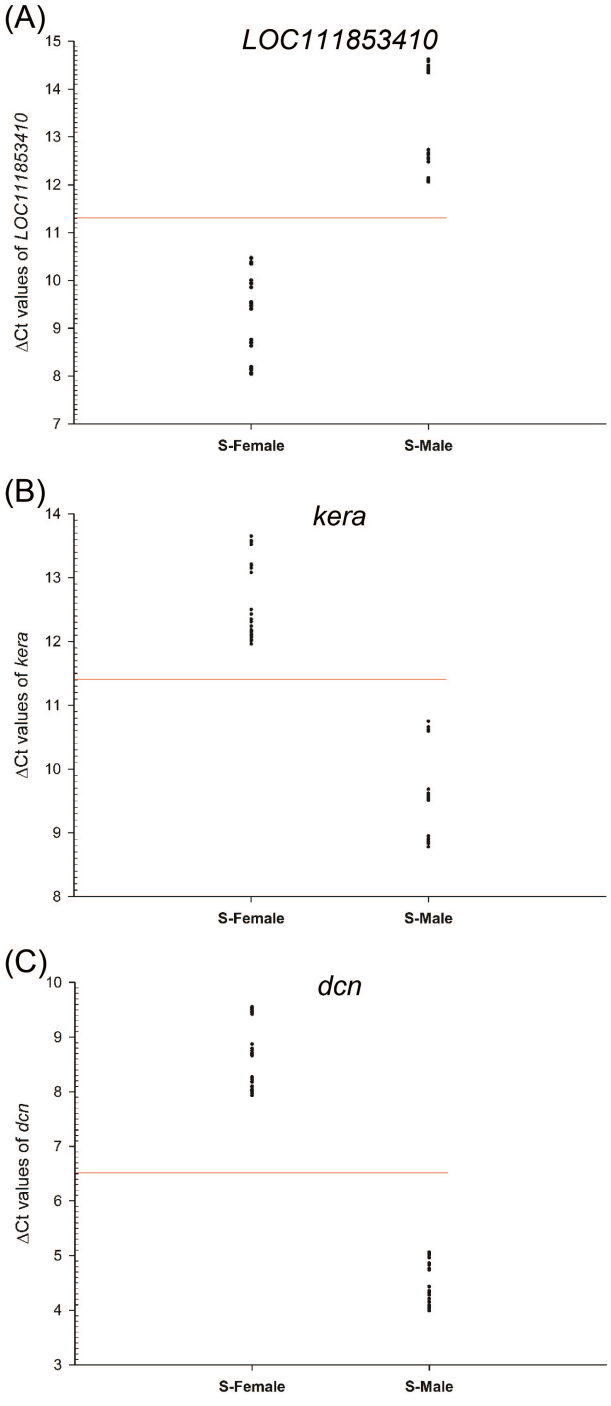

Fig. 4. Comparisons of ΔC_T values of *LOC111853410*, *kera*, and *dcn* between the "S-Female" (n = 7, four repeats) and "S-Male" (n = 5, four repeats) groups for assessing the threshold for sex typing. (A) *LOC111853410*, (B) *kera*, (C) *dcn*. A solid black circle represented one ΔC_T value. The "S-Female" meant the female silver eels, and the "S-Male" meant the male silver eels. For each gene, there were 28 and 20 solid black circles in the "S-Female" group and the "S-Male" group, respectively. The solid red line meant the inferred threshold of ΔC_T for sex typing.

scale production of young Japanese eels (glass eels). With the rapid development and popularization of next-generation sequencing, the use of transcriptomic analysis in the investigation of physiological mechanisms has been adopted by an increasing number of researchers (Dirks et al. 2014; Geffroy et al. 2016; Hsu et al. 2015; Huang et al. 2021). In this study, partial pectoral fin tissues of both female and male silver eels were sampled to permit RNA-seq. The transcriptomic data that were acquired were subjected to a comparative transcriptomic analysis performed on our online database. This database is a powerful tool that can accelerate genomic and transcriptomic studies on various signaling transduction pathways, physiological mechanisms, and metabolic pathways in eels. The genome of the Japanese eel has not been completely assembled, as reported in previous studies, because of teleost-specific genome duplication, which increases the complexity and difficulty of assembling a complete genome from small DNA fragments (Chen et al. 2019; Henkel et al. 2012). However, our previous genome assembly project resulted in sequencing of the genome of the Japanese eel with > 98% integrity. A gene model containing 27,350 genes was established by integration and comparison with multiple transcriptomic data. After annotation by the NR database, most of these genes corresponded to the homologous genes of fish species belonging to Actinopterygii, most of which are freshwater species and species that migrate between rivers and seas.

Many sexually dimorphic genes have been identified in the brain and gonadal tissues in previous studies. These studies mainly focused on the mechanisms of sex determination and differentiation (Geffroy et al. 2016; Inaba et al. 2021; Jeng et al. 2018). In this study, we aimed to assess the sex of Japanese silver eels before artificial maturation using sexually dimorphic genes found in tissues other than the brain and gonads, which are difficult to collect without harming the eels. Consequently, identifying sexually dimorphic genes in the pectoral fin for sex typing is an alternative method. A similar research strategy has been reported in which the molecular markers in the pectoral fin were identified to assess the gonadal development of female European eels induced by hormones during artificial maturation (Dirks et al. 2014). In our study, the analytic results of transcriptomic data indicated that a total of 29 candidates of sexually dimorphic genes in the pectoral fins of the Japanese silver eels were discovered, of which 15 genes were highly expressed in females (at least six times higher than that in males) and 14 genes were highly expressed in males (at least six times higher than that in females). Ten of the 29 candidate genes (*LOC108249696*, *LOC110515957*,

egr3, *cipc*, *LOC111853410*, *cttn*, *kera*, *dcn*, *cited1*, and *LOC104575574*) were selected for RT-qPCR to evaluate their feasibility as markers because they had more complete coding sequences and more detailed annotations.

RT-qPCR results of the female and male silver eels revealed that the expression of *LOC108249696*, *LOC110515957*, *egr3*, *cipc*, *cttn*, *cited1*, and *LOC104575574* was not completely consistent with the findings of the comparative transcriptomic analysis, and implied that these genes were not suitable markers for assessing the sex of Japanese eels. In addition, among these seven genes, variation in the expression level of each gene in different silver eels may be due to individual differences resulting from genetics, environmental stimulation, and physiological conditions. Another point worth noting was that the expression level of *LOC108249696* was significantly lower in the feminized eels than in other silver eels. *LOC108249696* encodes a septin 2-like protein belonging to an evolutionarily conserved family of GTP-binding proteins (Damalio et al. 2012). Septin proteins may be involved in cytokinesis, apoptosis, innate immunity, neurodegeneration, and neoplasia in fish and most are expressed in mucosal tissues to resist pathogen infection (Fu et al. 2016; Mazon-Moya et al. 2017). Whether the lower expression of *LOC108249696* in feminized eels can lead to weaker disease resistance requires further investigation.

By contrast, the results of transcriptomic analysis and RT-qPCR indicated that *LOC111853410*, *kera*, and *dcn* were differentially expressed in the pectoral fins of female and male silver eels. The expression level of *LOC111853410* was higher in female pectoral fins than in male pectoral fins. However, *kera* and *dcn* expression was significantly lower in female pectoral fins than in male pectoral fins. Due to the expression differences between the female and male silver eel groups, *LOC111853410*, *kera*, and *dcn* may be sexually dimorphic genes used as markers for sex typing of Japanese silver eels. In this study, the ΔC_T values of *LOC111853410*, *kera*, and *dcn* from the pectoral fins of all silver eels were analyzed to infer the thresholds for defining the criteria for sex typing. The inferred thresholds of ΔC_T of *LOC111853410*, *kera*, and *dcn* were 11.3, 11.4, and 6.5, respectively, which may be favorable criteria for the sex typing of silver eels. Based on the Vertical Point Plot for every marker gene, these thresholds can clearly distinguish the sex of silver eels. We suggested that if a silver eel has a pectoral fin with a ΔC_T of *LOC111853410* < 11.3, a ΔC_T of *kera* > 11.4, or a ΔC_T of *dcn* > 6.5, it can be assessed as a female. However, if it has a pectoral fin with a ΔC_T of *LOC111853410* > 11.3, a ΔC_T of *kera* < 11.4, or a ΔC_T of *dcn* < 6.5, it can be assessed as a male.

This study further tested the feasibility of candidate genes applied to sex typing for juvenile eels. Unfortunately, in this study, we could only collect the pectoral fins of male eels in juveniles. Then, ten candidate genes obtained from the pectoral fins of juvenile male eels were subjected to RT-qPCR. The RT-qPCR results showed that *LOC111853410*, *kera*, and *dcn* may also be markers for the sex typing of juvenile eels. Based on the Vertical Point Plot of every marker for adult and juvenile male eels, we thought that the inferred thresholds of ΔC_T of *LOC111853410* (11.3), *kera* (11.4), and *dcn* (6.5) from adult eels could also be applicable to juvenile eels for sex typing. All the pectoral fins of juvenile male eels had a ΔC_T of *LOC111853410* > 11.3, a ΔC_T of *kera* < 11.4, and a ΔC_T of *dcn* < 6.5, which are consistent with the results of adult male eels. As a result, if a juvenile eel has a pectoral fin with a ΔC_T of *LOC111853410* > 11.3, a ΔC_T of *kera* < 11.4, or the ΔC_T of *dcn* < 6.5, it can also be assessed as a male. This is a significant discovery that will be of great help in the sex identification of Japanese eels. In the future, the pectoral fins of juvenile female eels shall be further collected for RT-qPCR to verify and strengthen the feasibility of the inferred thresholds of ΔC_T of *LOC111853410*, *kera*, and *dcn* applied to the sex typing of Japanese eels.

LOC111853410 encodes a MUC19-like protein, a secreted mucin (gel-forming) that is extensively found in numerous tissues, such as the skin, gills, and the gastrointestinal tract of fish (Bai et al. 2020; Davey et al. 2011; Long et al. 2013; Sharba et al. 2022). Fish skin mucins play an important role in handling environmental stress and in maintaining the mucosal immune system (Davey et al. 2011; Long et al. 2013; Marel et al. 2012; Perez-Sanchez et al. 2013). The present results reveal that the MUC19-like protein is expressed in the pectoral fins of Japanese silver eels and is more highly expressed in females (especially in feminized eels) than in males, implying that the capacities of environmental adaptation and skin immunity may be better in females. Further studies on the skin of Japanese eels are needed to prove this inference.

The *kera* and *dcn* genes encode keratocan (keratan sulfate-containing proteoglycan) and decorin precursor-like protein, respectively. Both proteins belong to the small leucine repeat proteoglycan family (Iozzo 1999). Keratocan can facilitate the formation of uniform collagen in the extracellular matrix, modulate hydration of corneal stroma, and regulate corneal transparency in mammalian animals (Chakravarti 2001; Ernst et al. 2000; Iozzo 1999). Additionally, keratocan has been associated with an inherited human disease, corneal plana, which manifests as reduced visual acuity,

flattened corneal curvature, and thin corneal stroma (Pellegata et al. 2000). Zebrafish have been used as a model system for studying corneal development and disease. Results indicate that *zKera* may have a critical function in the normal corneal and embryonic development of zebrafish (Yeh et al. 2008). Decorin can directly bind to type I collagen, a key biological interaction that controls the pace and extent of collagen fibril formation (Reed and Iozzo 2002). In addition to type I collagen, decorin binds to zinc ion, fibronectin, thrombospondin, transforming growth factor beta, low-density lipoprotein receptor related protein 1, and epidermal growth factor receptor (Brandan et al. 2008; Ferdous et al. 2007; Hildebrand et al. 1994; Iozzo et al. 1999; Schmidt et al. 1987; Winnemoller et al. 1992; Yang et al. 1999). Moreover, decorin has also been reported to be involved in the pathogenesis of renal diseases (Schaefer et al. 2004), cancer (Reed et al. 2005; Seidler et al. 2006), angiogenesis (Grant et al. 2002), wound healing (Jarvelainen et al. 2006), myocardial infarction (Weis et al. 2005), tooth development (Goldberg et al. 2005), and bone marrow stromal cell biology (Bi et al. 2005). A study on zebrafish showed that decorin protein plays an important role during medial-lateral and anterior-posterior extensions of the body plan, as well as in craniofacial cartilage formation (Zoeller et al. 2009). However, there is almost no information on the functions of keratocan and decorin in Japanese eels.

The functions of keratocan and decorin proteins in Japanese eels warrant further investigation, and the biological significance of the expressional differences of *kera* and *dcn* in pectoral fin tissues between female and male silver eels also needs to be explored. In addition, *kera* and *dcn* are highly expressed in pectoral fin tissues of male yellow eels, which is a very interesting and meaningful finding and needs more investigation. The scales or pectoral fins of Japanese eels might also be a unique biological model for fabricating materials, such as keratocan and decorin proteins, for corneal stromal regeneration (Takagi and Ura 2007).

CONCLUSIONS

Overall, the research strategy of this study proved feasible. Three candidate sexually dimorphic genes, *LOC111853410*, *kera*, and *dcn*, in pectoral fin tissue may be potential markers for sex typing. *LOC111853410* displayed higher expression levels in female silver eels than in male silver eels, while the expression levels of *kera* and *dcn* were higher in males than in females. We suggested that if a silver eel has a pectoral fin with a ΔC_T of *LOC111853410* < 11.3, a ΔC_T of *kera* > 11.4, or a ΔC_T of *dcn* > 6.5, it can be assessed as a female. In addition, a silver or yellow eel can be assessed as a male if it has a pectoral fin with a ΔC_T of *LOC111853410* > 11.3, a ΔC_T of *kera* < 11.4, or a ΔC_T of *dcn* < 6.5. In the future, the possible value of the remaining 19 candidates as markers will be examined, and the molecular functions of these markers in Japanese eels will be explored. These markers should also be assessed in relation to sexual maturation, which could clarify the sexual maturation process in eels.

List of abbreviations

RT-qPCR, Real-time quantitative polymerase chain reaction.
RNA-seq, RNA-sequencing.
GTF, Gene Transfer Format.
KEGG, Kyoto Encyclopedia of Genes and Genomes.
FPKM, Fragments per kilobase of exon per million fragments mapped.
C_T, cycle threshold.
arp, acidic ribosomal phosphoprotein P0.
SD, standard deviation.
bp, base pair.
egr3, early growth response protein 3.
cipc, CLOCK-interacting pacemaker.
cttn, src substrate cortactin.
kera, keratocan.
dcn, decorin.
cited1, cbp/p300-interacting transactivator 1.

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Authors' contributions: HYH was the first author and conducted the experiments, analyzed the research data, performed the statistical analysis, and wrote the manuscript. CHC, IHL, and CYL assisted in assembling the Japanese eel genome, analyzing transcriptomic data, and establishing an online database. YSH designed and supervised the experiments and participated in the manuscript writing and interpretation of the results.

Competing interests: HYH, CHC, IHL, CYL, and YSH declare no competing interests.

Availability of data and materials: All the data

are provided within this manuscript (see supplementary materials).

Consent for publication: Not applicable.

Ethics approval consent to participate: All procedures and investigations were approved by the Institutional Animal Care and Use Committee of the National Taiwan University (NTU107-EL-00059) and executed in accordance with standard guiding principles.

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Supplementary materials

Table S1. Detailed information of Japanese silver eels used in this study. (download)

Table S2. Detailed information of Japanese yellow eels used in this study. (download)

Table S3. Data of NGS statistics and mapping rate to the genome of Japanese eel for each library. (download)

Table S4. Coding DNA sequences of the 29 transcripts which are candidate sexually dimorphic genes in the pectoral fin of Japanese eel. (download)

Fig. S1. Expression profiles of six genes, possibly having higher expression in the pectoral fin tissues of female silver eels, were obtained from silver and yellow eel samples by RT-qPCR. (A) *LOC108249696*, (B) *LOC110515957*, (C) *egr3*, (D) *cipc*, (E) *LOC111853410*, (F) *cttn*. The *arp* was a housekeeping gene, and two female samples (F1 and F2) were used as the internal control for calculating relative expression levels. The data were presented as means ± SD, and the y-axis was in $\log_2$ scale. Different letters indicate significant differences ($P < 0.05$) in gene expression among the pectoral fin tissues of Japanese eel samples. For the silver eels, F meant the female, and M meant the male. Additionally, Y-M meant the male yellow eels. (download)

Fig. S2. Expression profiles of four genes, possibly having higher expression in the pectoral fin tissues of male silver eels, were obtained from silver and yellow eel samples by RT-qPCR. (A) *kera*, (B) *dcn*, (C) *cited1*, (D) *LOC104575574*. The *arp* was a housekeeping gene, and two female samples (F1 and F2) were used as the internal control for calculating relative expression levels. The data were presented as means ± SD, and the y-axis was in $\log_2$ scale. Different letters indicate significant differences ($P < 0.05$) in gene expression among the pectoral fin tissues of Japanese eel samples. For the silver eels, F meant the female, and M meant the male. Additionally, Y-M meant the male yellow eels. (download)

Fig. S3. Comparisons of ΔC_T values of *LOC111853410*, *kera*, and *dcn* between the "S-Male" ($n = 5$, four repeats) and "Y-Male" ($n = 10$, four repeats) groups for assessing whether the inferred threshold from silver eels is applicable to juvenile eels (yellow eels) for sex typing. (A) *LOC111853410*, (B) *kera*, (C) *dcn*. A solid black circle represented one ΔC_T value. The "S-Male" meant the male silver eels, and the "Y-Male" meant the male yellow eels. For each gene, there were 20 and 40 solid black circles in the "S-Male" group and the "Y-Male" group, respectively. The solid red line meant the inferred threshold of ΔC_T from silver eels for sex typing. (download)