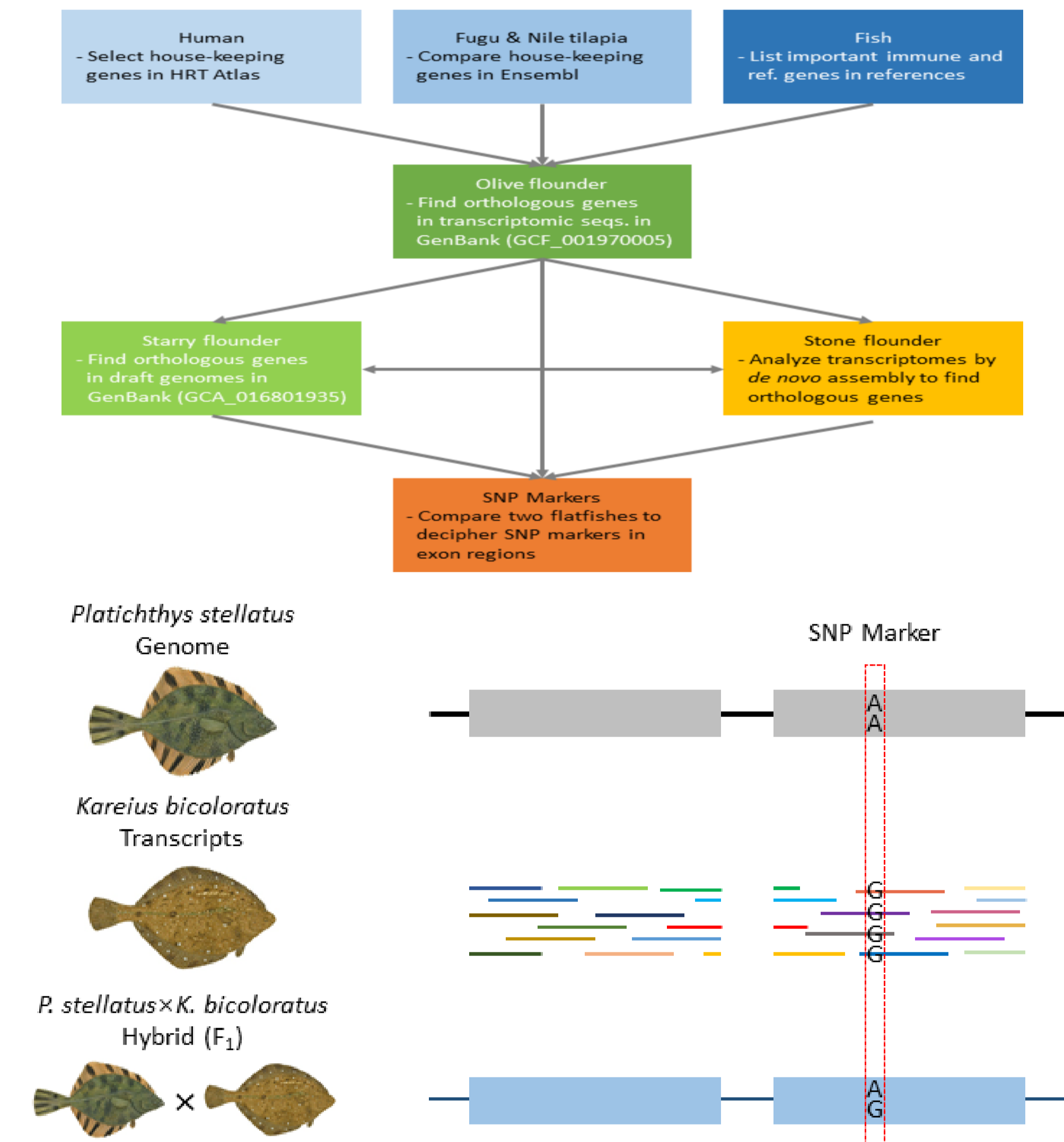


## Abstract

The advancements in aquaculture techniques, including hybridization, selective breeding, and backcrossing, have substantially enhanced genetic and productive traits. However, morphological classification poses challenges in identifying hybrid flounders. Thus, there is a pressing need to develop an analytical method for precise species discrimination to ensure accurate and secure production and research endeavors in the future. To address this, genotypic verification samples comprised starry flounder, stone flounder, starry flounder (♀) × stone flounder (♂) = hybrid (F<sub>1</sub>), hybrid (F<sub>1</sub>) (♀) × starry flounder (♂), and hybrid (F<sub>1</sub>) × stone flounder (♂) backcrosses. Over 50 candidate genes from various sources (Human, Fugu, Nile tilapia, Fish, Olive flounder, Starry flounder, and Stone Flounder) were analyzed to identify Single Nucleotide Polymorphism (SNP) molecular markers capable of distinguishing between starry and stone flounders. Through genomic analysis, 32 SNP molecular markers were selected and subjected to Kompetitive allele specific PCR (KASP®) genotyping. Ultimately, 16 SNP molecular markers (*CSNK2B*, *EDF1*, *HNRNPA2B1*, *MLF2*, *eef1a1*, *egr1*, *gpx1a*, *gpx4b*, *igf1*, *lyg2*, *myh6*, *pitx2*, *rag1*, *rho*, *rp17*, and *txn1*) were identified as capable of distinguishing between hybrid and backcross generations. This method offers a robust species classification technology essential for effective aquaculture management and genetic research.

## Material & Method

### SNP molecular marker discovery strategy



### Design of KASP molecular marker

A SNP region that can identify the species of starry flounder and stone flounder based on the base sequence alignment information of the exon region of each candidate gene inferred by comparing the genome information of puffer fish, Nile tilapia, olive flounder, and starry flounder with the transcriptome information of sea flounder. was explored. Based on this, 35 candidate genes for KASP genotyping were selected, including one or more SNPs capable of species identification of starry flounder and stone flounder and their surrounding regions. Afterwards, the DNA sequence information of each gene was prepared according to KASP Assay Design (LGC, UK), and KASP Assay Mix for KASP genotyping was commissioned and produced by LGC.

## Results

### Selection of candidate genes and securing base sequences

Table 1. List of candidate genes that are stably expressed in all tissues of vertebrates and humans

No.	Gene name	Full gene name
01	AES	TLE family member 5, transcriptional modulator
02	AP2M1	adaptor related protein complex 2 subunit mu 1
03	BSG	basigin
04	CSNK2B	casein kinase 2 beta
05	EDF1	endothelial differentiation related factor 1
06	EEF2	eukaryotic translation elongation factor 2
07	HNRNPA2B1	heterogeneous nuclear ribonucleoprotein A2/B1
08	HSP90AB1	heat shock protein 90 alpha family class B member 1
09	MLF2	myeloid leukemia factor 2
10	PFDN5	prefoldin subunit 5
11	PSAP	prosaposin
12	RAB11B	RAB11B, member RAS oncogene family
13	RAB1B	RAB1B, member RAS oncogene family
14	RAB7A	RAB7A, member RAS oncogene family
15	ARF5	ADP ribosylation factor 5
16	RHOA	ras homolog family member A

## Results

### KASP analysis and Genotyping

The results were produced by performing KASP genotyping on three representative samples each of starry flounder and stone flounder, their hybrids, and the backcross generation using the produced molecular marker for genotyping.

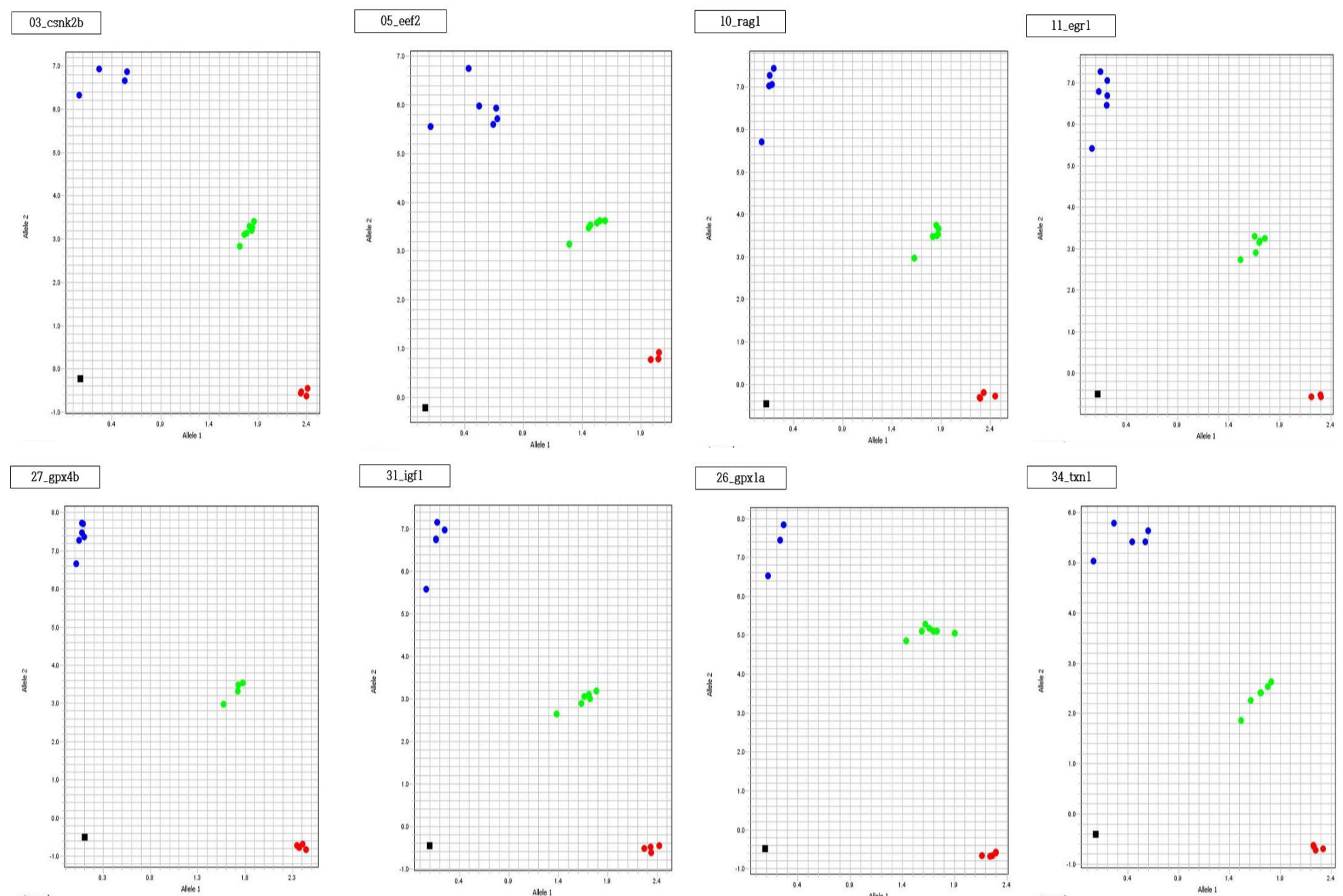


Fig. 2 Allelic Discrimination Plot produced by performing KASP genotyping on representative samples of starry flounder and stone flounder, their hybrids, and backcross generations.

Results of KASP genotyping of 13 human house-keeping genes and 28 fish-expressed genes selected for three representative samples each of starry flounder and stone flounder, their hybrids, and the backcross generation, resulting in the final selection of 16 genes.

Species	CSNK2B	EEF2	HNRNPA2	MLF2	rag1	egr1	my6	rho	eef1f1	rpl7	pitx2	lyg2	gpx1a	gpx4b	igf1	txn1
Ps01	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ps02	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Ps03	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Kb01	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Kb02	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Kb03	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
PsKb01	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
PsKb02	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
PsKb03	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
F1Ps01	3	1	3	3	3	1	1	3	1	3	1	1	3	1	3	1
F1Ps02	1	1	3	1	1	1	3	3	1	3	1	1	3	1	1	3
F1Ps03	3	1	1	3	1	1	1	1	1	1	1	1	3	1	1	1
F1Kb01	2	3	2	2	3	3	3	2	2	3	3	2	2	3	2	3
F1Kb02	3	3	2	3	3	3	3	2	2	2	3	2	3	2	3	3
F1Kb03	3	3	3	3	2	3	3	3	2	2	2	3	2	2	3	2

Fig. 3 The results of KASP genotyping of the finally selected human house-keeping genes and fish expressed genes on three representative samples each of the starry flounder (*Platichthys stellatus*, Ps) and stone flounder (*Kareius bicoloratus*, Kb), hybrid and backcross generations.

Table 2. Using the finally selected genes, KASP® genotyping was performed on the starry flounder (*Platichthys stellatus*, Ps) and stone flounder (*Kareius bicoloratus*, Kb), hybrid (PsKb) and backcross (F1Ps and F1Kb) generations, and species and generation were determined.

NO.	Species	Ps	Kb	PsKb	Ps	Kb	PsKb	Identification
1	Ps01	16	0	0	1.0000	0.0000	0.0000	Ps
2	Ps02	16	0	0	1.0000	0.0000	0.0000	Ps
3	Ps03	16	0	0	1.0000	0.0000	0.0000	Ps
4	Kb01	0	16	0	0.0000	1.0000	0.0000	Kb
5	Kb02	0	16	0	0.0000	1.0000	0.0000	Kb
6	Kb03	0	16	0	0.0000	1.0000	0.0000	Kb
7	PsKb01	0	0	16	0.0000	0.0000	1.0000	F <sub>1</sub>
8	PsKb02	0	0	16	0.0000	0.0000	1.0000	F <sub>1</sub>
9	PsKb03	0	0	16	0.0000	0.0000	1.0000	F <sub>1</sub>
10	F1Ps01	8	0	8	0.5000	0.0000	0.5000	F <sub>1</sub> Ps
11	F1Ps02	10	0	6	0.6250	0.0000	0.3750	F <sub>1</sub> Ps
12	F1Ps03	13	0	3	0.8125	0.0000	0.1875	F <sub>1</sub> Ps
13	F1Kb01	0	8	8	0.0000	0.5000	0.5000	F <sub>1</sub> Kb
14	F1Kb02	0	6	10	0.0000	0.3750	0.6250	F <sub>1</sub> Kb
15	F1Kb03	0	7	9	0.0000	0.4375	0.5625	F <sub>1</sub> Kb

## Conclusion

In conclusion, based on the results of the KASP genotyping analysis, the hybrid generation shows heterozygous alleles that are unique to the starry flounder and stone flounders, and the hybrid generation shows heterozygous alleles that carry their genes together. 16 genes that were useful for genotyping of straight cross, hybrids, and backcross generations were finally selected to determine species and generation.

## Acknowledgement

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