生殖細胞移植によるサバ類代理親魚技法の開発

博士学位論文

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(2022 年 3 月)

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第 1 章

海産魚における生殖細胞移植

緒言

代理親魚技法は、ナーとなる個体の生殖腺に存在する卵や精子のもとになる細胞である“生殖幹細胞” を宿主魚に移植し、この魚を成熟させることで、宿主魚にドナー由来の卵あるいは精子を生産させる技術である（Yoshizaki and Yazawa, 2019）。Okutsu ら（2007）は、ニジマス *Oncorhynchus mykiss* の生殖幹細胞を、ニジマスの近縁種であるヤマメ *Oncorhynchus masou* 仔魚の腹腔内へ移植することで、成熟したヤマメ代理親にドナー由来のニジマス配偶子を生産させることに成功した。この研究を皮切りに、我が国のみならず世界各国において、様々な魚種を対象として代理親魚技術を利用した研究へと展開されている（Jin et al., 2021）。海産の養殖対象魚種へも応用されており、アジ科（Morita et al., 2012, 2015）、フグ科（Hamasaki et al., 2017, Yoshikawa et al., 2018b）、ニベ科（Yoshikawa et al. 2017, 2018a）においてドナー由来の配偶子を生産する宿主魚の作出に成功している。またサバ科魚類においても、マサバを宿主魚として用いた基本的な精原細胞移植法が確立され、ドナー生殖細胞を宿主であるマサバの生殖腺に高効率で取り込ませることに成功しているが（Yazawa et al, 2010, 2013）、ドナー由来の配偶子生産には至っていない。

マサバ（*Scomber japonicas*）は、我が国周辺に広く分布する水産上重要種であり、一般的にはいわゆる大衆魚であるが、一部にはブランド魚として確立され非常に高価格で取引される例も見られる。また近年では、特に需要の高い大型魚の漁獲が不安定となるのに伴い、西日本を中心に養殖が盛んになっている（Mendiola et al., 2008; Hashimoto et al., 2019）。養殖マサバの特色として、まず高品質の魚を安定して供給できる点が挙げられる。天然のマサバでは、産卵期である春季以降から夏季にかけては痩せて脂も少ない一方で、秋季から冬季にかけては脂のりが良くなり明確な「旬」が存在し、旬の魚か否かで市場における評価が大きく分かれる。また、その漁獲量は、季節、年によって大きく変動するため、供給も不安定である。一方、

養殖マサバは給餌量や飼料成分、飼育環境のコントロール等により年間を通じて脂のりの良好な個体を安定的に出荷することが可能である。次に、刺身商材として利用可能であることも養殖マサバの大きなメリットである。サバ類はハンドリングに弱く傷みやすいことから、天然魚の活魚での流通は困難であるが、養殖魚では、マサバに特化したオペレーションによってスレを最小限にとどめ、活魚での出荷も可能となっている。さらに、天然サバでは、食物連鎖に起因するアニサキスが可食部に寄生するため、生食では健康被害のリスクが極めて高い。一方、養殖では、餌飼料の管理によってアニサキス感染のリスクが極めて低いことから刺身商材として利用可能である。このような背景から、養殖マサバをブランド化、地域特産化する試みが日本各地で行われており、天然ブランドサバにも匹敵する付加価値の高い養殖マサバも登場している。

今後、効率的かつ持続的なサバの養殖を実現するために、品種改良による優良系統の確立が求められている。サバ類の成熟誘導技術や完全養殖はすでに確立されており（Matsuyama et al., 2005; Murata et al., 2005; Shiraishi et al., 2005）、選抜育種のための基盤技術が存在する。従来の選抜育種では、高成長や耐病性などの優良な形質を持つ個体を選抜し、これらの個体同士の交配を繰り返す必要があった。しかし、サバ科魚類ではグループ産卵を行うため、選抜個体を雌雄同時に催熟し、１：１交配により次世代を得ることは容易ではない。また、サバ科魚類では仔稚魚期の生残性が低いことや、感染症による斃死、自然災害や事故など、選抜個体を失うリスクも存在し、これらが育種における大きな課題となっている。そこで、代理親魚技法を利用することで、これらの課題を克服できると考えた。すなわち、優良形質を保持した個体から得た生殖幹細胞を多くの宿主個体へ移植することで、ドナーである優良個体由来の配偶子をつくる雌雄親魚を大量に生産することで、選抜個体を失うリスクを大幅に低減することが可能になる。また、得られた代理親魚集団を同一水槽内でグループ産卵させることにより、優良個体に由来する雌雄親魚同士の F

１世代を効率的かつ確実に生産することが可能となる。

また、サバ類育種によって優良系統を確立できた場合の新たな課題として、系統の維持にも考慮する必要がある。系統維持のためには、優良形質を保持する親魚を生体として維持しながら、毎世代ごとに計画的な交配を行っていく必要があり、そのためには多くのコストやスペース、労力が必要となるうえ、上述した個体損失リスクは変わらず存在する。この課題を解決する方法として、凍結した生殖幹細胞を用いた生殖細胞移植による次世代の生産技術が挙げられる。すでに複数の魚種において、ドナー個体の生殖細胞を液体窒素で長期冷凍保存したのちに解凍し、宿主個体へと移植することで、ドナー生殖細胞に由来する生きた魚を生産できることが示されている（Lee et al., 2013； Yoshizaki & Lee、2018）。したがって、十分に育種の進んだ優良系統の生殖細胞を凍結保存することが出来れば、必ずしも生体を維持して交配を繰り返す必要がなくなるうえ、前述したリスクによりその系統の生体が失われたとしても、代理親魚技法を用いることで復活が可能となる。以上のように、サバ類において生殖細胞移植による代理親魚技法が確立されれば、サバ類養殖に大きく貢献可能であると考えた。

前述の通り、Okutsu ら（2007）は、ヤマメを代理親魚として用い、ニジマスの機能的な配偶子を生産することに成功した。この際、ドナー由来の配偶子を効率的に生産するために、宿主を不妊化することが極めて重要である。通常の妊性を有するヤマメにニジマス細胞を移植した場合、宿主が生産する配偶子には、ドナー由来の配偶子に加え、宿主であるヤマメ自身の配偶子が混在する。Okutsu ら（2007）は、三倍体化処理による不妊化ヤマメを宿主とすることで、ドナー由来であるニジマスの配偶子のみを獲得することを実現している。宿主を不妊化する手法としては、受精卵への温度･圧力処理による三倍体化（Okutsu et al., 2007; Yoshizaki et al., 2010; Lee et al., 2013; Yoshikawa et al., 2017; Hamasaki et al., 2017; Seki et al., 2017）や、生殖細胞の生存に必須な遺伝子である *dead end* 遺伝子（Weidinger et al., 2003）の機能阻

害が不妊化法として利用されてきた（Saito et al., 2008; Yoshizaki et al., 2016; Li et al., 2017; Octavera and Yoshizaki, 2019; Marinović et al., 2019）。しかし、現在までにサバ科魚類において安定的かつ大量に不妊魚を作出可能な技術の開発には至っていない。それは、これら不妊化方法が受精卵への処理を必要とし、宿主の生残に多大な影響を与えることから、もともと初期減耗が著しいサバ魚類においてこれらの手法を用いて不妊宿主の大量生産は非常に困難であることが大きな原因の一つである。

そこで、本研究では、Kawamura ら（2020）によって報告された、ゴマサバ雌とマサバ雄の交配で得られる F 1 世代の種間交雑サバ（ゴママサバ雑種）を宿主として用いることを考えた。たサバ類代理親魚技法の確立を試みた。Kawamura ら（2020）によると、81.8%の個体がが生殖細胞欠損型の不妊であり、かつ移植されたドナー由来生殖細胞の維持に不可欠な生殖腺における支持細胞とステロイド産生細胞は保持していることが確認されている。実際に、ゴママサバ雑種の腹腔内に移植されたドナー生殖細胞が、高い効率で生殖腺に取り込まれたことも確認された（Kawamura et al., 2020）。しかし、ドナー由来の配偶子が形成されるかどうかは未だ確認されていない。そこで本研究では、ゴママサバ雑種を宿主とした生殖細胞移植を行い、ドナー由来の配偶子の生産を目指した。

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第 2 章

生殖細胞移植によるサバ類代理親魚技法の開発

Introduction

Surrogate broodstock technology consists of producing donor-derived gametes in a surrogate fish by transplanting germline stem cells of a donor to a recipient of a different strain or species (Yoshizaki and Yazawa, 2019). Intraperitoneally transplanted donor germline stem cells migrate towards recipient genital ridges by chemotaxis and are eventually incorporated. The donor-derived germline stem cells start either spermatogenesis or oogenesis depending on the sex of the recipient fish (Yoshizaki and Lee, 2018). Using this technique, we successfully generated masu salmon (*Oncorhynchus masou*) that produce gametes of rainbow trout (*O. mykiss*) (Takeuchi et al., 2004; Okutsu et al., 2007; Lee et al., 2015; Yoshizaki et al., 2016). As an application of the surrogate broodstock technology in aquaculture, seed production of large-bodied and commercially important fish species would be possible in small land-based fish tanks using surrogate small-bodied parents with short generation times. Thus, we have attempted to establish a small-bodied surrogate recipient, such as mackerel, that produces functional bluefin tuna gametes. If realized, the strategy will save space, labor and cost for the seedling production of bluefin tuna. To date, surrogate recipients that produce allogeneic and xenogeneic gametes is established in various marine teleosts, including Carangidae (Morita et al., 2012, 2015), Tetraodontidae (Hamasaki et al., 2017, Yoshikawa et al., 2018b) and Sciaenidae (Yoshikawa et al., 2017, 2018a). We also developed a spermatogonial transplantation technique using chub mackerel as recipients

(Yazawa et al., 2010, 2013).

To establish a surrogate broodstock for bluefin tuna, sterile mackerel recipients are desirable. When reproductively fertile fish are used as recipients, endogenous gametogenesis outcompetes donor-derived gametogenesis, resulting in dominant production of recipient gametes. We previously demonstrated that triploid recipients can produce only donor-derived gametes (Okutsu et al., 2007; Yoshizaki et al., 2010; Lee et al., 2013; Yoshikawa et al., 2017; Hamasaki et al., 2017; Seki et al., 2017). As an option to triploidization, sterile recipients lacking endogenous germ cells caused by gene knockdown or knockout of the *dead end* (*dnd*) gene, which is required for the maintenance of primordial germ cells (Weidinger et al., 2003), has been used as recipients in several previous reports (Saito et al., 2008; Yoshizaki et al., 2016; Li et al., 2017; Octavera and Yoshizaki, 2019; Marinović et al., 2019).

However, application of these sterilization methods to marine fish is difficult due to their generally high mortality during larval development. Since the above-mentioned methods directly treat fertilized eggs, production of large numbers of sterile fish with is unrealistic with feeble marine fish larvae. Although triploidy in eastern little tuna (*Euthynnus affinis*) is successfully induced, triploid fish showed high mortality than the diploids during two to four weeks post-hatching because of selective cannibalism by diploid siblings (Yazawa et al., 2019). Thus, an alternative method suitable for marine fish is required for the mass-

production of sterile recipient fish.

In this study, we focused on interspecific hybridization as an alternative approach. Hybrid sterility is a common phenomenon observed in many eukaryotic inter-species hybrids, including examples in yeast, plants, insects, birds, and mammals (Coyne and Orr, 2004; Maheshwari and Barbash, 2011). Interspecific hybridization is commonly used in the field of aquaculture to improve economic value. Typically, combinations of superior traits of parent species and positive heterosis are sought, and numerous examples of sterile hybrids are known for various fish species (Bartley et al., 2001; Rahman et al., 2013). Further, several reports that used sterile hybrids as recipients for germ cell transplantation (Yamaha et al., 2003; Shimada and Takeda, 2008; Wong et al., 2011; Xu et al., 2019). Recently, we found that hybrids of female blue dram (*Nibea mitsukurii*) × male white croaker (*Pennahia argentata*) display germ cell-less sterile gonads (Yoshikawa et al., 2018a). Importantly, these hybrid recipients receiving blue dram spermatogonia, produced only donor-derived gametes (Yoshikawa et al., 2018a). These examples of use of hybrid recipients indicate that sterile hybrids can fully support gametogenesis of transplanted germ cells. Thus, interspecific hybridization can be an effective method to produce sterile recipients. Moreover, interspecific hybridization enables mass-production of recipient fish simply by *in vitro* insemination and requires no additional treatment of fertilized eggs.

We focused on blue mackerel (*Scomber australasicus*) and chub mackerel, belonging to

Scombridae to produce sterile hybrids for the creation of broodstock for bluefin tuna. Since both mackerel species are distributed in the coastal waters of Japan, and their spawning areas and timing show a large overlap (Yukami et al., 2009), we expected that hybrid sterility may be the reason for reproductive isolation of these closely related species. In the present study, we produced hybrid mackerel using a combination of female blue and male chub mackerel and assessed gonadal development. Further, its suitability as surrogate broodstock to produce bluefin tuna gametes was evaluated.

Materials and methods

Spawning induction of broodstock

Blue mackerel and chub mackerel were caught in purse seines and held for a month in an offshore aquaculture net pen (144 m^2 × 5-m depth) located in Higashi-Matsuura, Saga prefecture, Japan. On December 2013, approximately 40 blue mackerel and 160 chub mackerel (400-600 g in body weight; 30-40 cm in fork length) were transferred to Tateyama Station (Banda), Field Science Center of the Tokyo University of Marine Science and Technology (34°97′N, 139°76′E). These broodstock fish were reared in a land-based 70-m^3 fiber reinforced plastic (FRP) circular tank with flow-through seawater (100 l/min) under a natural photoperiod until use. The broodstock were fed extruded pellets (White Ikusei no.4; Hayashikane Sangyo, Yamaguchi, Japan), defrosted sand eel, and krill to apparent satiety twice daily. On May 2014, 10 blue and chub mackerel were transferred from the 70-m^3 rearing tank to 5-m^3 FRP-tanks for spawning induction with flow-through seawater (25 l/min) at 20.5°C–21.2°C under a natural photoperiod. To induce final maturation of parental fish, gonadotrophin-releasing hormone agonist (GnRHa) for the spawning induction was administered by implantation of sustained-release cholesterol pellets (Amezawa et al., 2018). Cholesterol pellets containing GnRHa were prepared by a custom peptide synthesis service (Anygen, Jeollanam-do, Korea) and implanted into the abdominal cavity. Pellets contained GnRHa at a dose of 100 μg/kg BW.

Interspecific hybridization

Approximately 32 h after GnRHa treatment, the fish were anesthetized with 100 ppm. 2-phenoxyethanol (Fujifilm Wako Pure Chemical Co., Osaka, Japan). Ovulated eggs were obtained from female blue mackerel by gently squeezing its abdomen, and the eggs were collected into a 2,000 ml beaker. Approximately 1 ml of milt was collected from each chub mackerel male (for hybrid) and each blue mackerel male (for control blue mackerel) by pressing its abdomen. Milt was kept on ice until use. To compare the early development of hybrid mackerel and control blue mackerel, blue mackerel eggs were fertilized with chub mackerel or blue mackerel sperm. Blue mackerel eggs were divided into two fractions each containing approximately 40,000 eggs. One fraction was mixed with 1 ml of chub mackerel sperm, and the other with 1ml blue mackerel sperm. Mixed eggs and sperm were subsequently activated by adding 1,000 ml natural seawater and rinsed twice with natural seawater. Eggs that rose to the water surface within 5 min after fertilization were defined as floating eggs and triplicate lots of 20 floating eggs were collected into 8 ml Petri dishes filled with sterile seawater and incubated at 20°C. The number of eggs that reached the two- to four-cell stages within 1–2 h of fertilization was recorded as fertilized egg count, and the number of larvae that hatched within 48 h of fertilization was recorded as hatched egg count. The fertilization rate was calculated as [fertilized egg count / 20 floating eggs × 100], and

hatching rate calculated as [hatched larva count / 20 floating eggs × 100]. This cross experiment was repeated three times, using different batches of fertilized eggs.

Larval culture

Approximately 20,000 fertilized eggs of hybrid mackerel were transferred to a 100-l polycarbonate tank (440 mm diameter × 700 mm deep) with flow through seawater and maintained at 20°C–21°C with gentle aeration. Larval rearing was described previously (Yazawa et al. 2010). Briefly, feeding of larvae began 2 days post-hatch (dph). The rotifer *Brachionus rotundiformis*, fed with freshwater type of Chlorella (Super fresh Chlorella V12; Cholera Industry Co., LTD, Tokyo, Japan), was added to the tank twice a day. Densities of rotifers and *Nannochloropsis sp.* (Marine fresh; Marine-bio Inc., Kumamoto, Japan) in the tank were maintained at 10 individuals/ml and 5×10^5 cells/ml, respectively. *Artemia* nauplii and an artificial diet (Otohime, Pacific Trading Co., Ltd., Fukuoka, Japan) were provided from 12- and from 14-dph, respectively. To increase the n-3 fatty acid concentration in live food, the rotifers and *Artemia* nauplii were incubated with Hyper Gloss (Nissin Marine Tech Co. Ltd., Kanagawa, Japan) for 6 to 12 h before feeding. Fish were reared in 100-l tanks for approximately 25 days, followed by transfer into 5-m^3 tanks. The photoperiod and water temperature were not modified from ambient conditions.

Polymerase chain reaction (PCR) and ploidy analysis.

Aneuploid, gynogenic, or androgenic offspring were previously produced by interspecific hybridization (Liu, 2010). Therefore, to confirm successful production of diploid hybrids, 10 hybrid larvae at 1-dph were subjected to DNA analyses against nuclear DNA (nDNA) or mitochondrial DNA (mtDNA). Detection of parental nDNA was performed using a multiplex PCR kit “Saba checker-I” (SCOTS, Saga, Japan) according to manufacturer's instructions. This method amplified both blue and chub mackerel specific regions in the nuclear ribosomal DNA (rDNA) internal transcribed spacer (ITS1) region. Identification of maternal origin was performed by PCR-restriction fragment length polymorphism (RFLP) analysis of mtDNA following a previously reported protocol (Food and Agricultural Materials Inspection Center and Fisheries Research Agency, 2007). Briefly, the PCR was performed with primer sets LSs1-Leu and HSs1-ND5 (Table. 1), designated for the conserved region located from tRNA-Leu (CUN) to NADH dehydrogenase subunit 5 (ND5) gene between chub and blue mackerel. PCR amplification was conducted in a 50-μl reaction volume containing 1× PCR Buffer II, 200 μM of dNTPs, 1.5mM $MgCl_2$, 1.25 U of Ampli*Taq* Gold DNA polymerase (Thermo Fisher Scientific, MA, USA), 50 ng of template DNA, and 1 μM of each primer. Thermal cycling conditions were: 1 cycle of 95°C for 8 min, then 35 cycles of 94°C for 30 s, 55°C for 15 s, 72°C for 1 min, followed by a final elongation step at 72°C for 7 min. Amplified PCR fragments were digested for an hour at

37°C with *Hae* III, which recognizes a sequence unique to the target region of chub mackerel. PCR-RFLP products were electrophoresed on a 2.0% agarose gel. To estimate average cellular DNA contents of parental species and hybrid larvae at 1-dph, flow cytometric analysis was performed as described previously by Yazawa et al. (2019). Relative DNA content of each larva was measured using a Guava PCA-96 (Millipore, Billerica, MA). CyStain PI Absolute T kits (Partec, Munster, Germany) were used according to the manufacturer's instructions and a blue mackerel larva was used to represent the standard DNA content value of respective sample types. Flow cytometry was performed using ten 1-dph hybrid mackerel, control chub mackerel, and blue mackerel.

Histological analysis of hybrid mackerel gonads

Fish used in histological analyses were 30-, 60-, 120-dph, 1 and 2-year-old hybrid mackerel. Gonadosomatic indices (GSI; [gonad weight in grams/body weight in grams] × 100) were measured to monitor gonadal development of hybrid mackerel. Gonads were fixed with Bouin's fixative overnight at 4°C, cut into 4-μm thick sections using standard paraffin-embedding methods, and stained with hematoxylin and eosin. Images of sections were obtained using a light microscope (BX-51; Olympus, Tokyo, Japan) and a digital camera (DP-70; Olympus). This histological analysis was performed using at least 10 gonads of hybrid mackerel at each age.

Gene expression analyses of hybrid mackerel gonads

The localization of germ cell marker, DEAD-box polypeptide 4 (*ddx4*) mRNA, and Sertoli cell marker, gonadal soma derived growth factor (*gsdf*) mRNA, were analyzed by in situ hybridization (ISH) on tissue sections of 120-dph hybrid and blue mackerel. Antisense RNA probes were synthesized from 379-bp chub mackerel *ddx4* (nucleotide 2,008-2,387 bp; GQ404693), and 488-bp chub mackerel *gsdf* (nucleotide 1-488 bp; GQ404694) cDNA fragments, as previously described (Yazawa et al., 2010). The ISH was performed as described previously (Sawatari et al., 2007). Since homologies of *ddx4* and *gsdf* probes between the two mackerel are 95.2% and 98.7%, respectively, these probes are expected to be hybridized to transcripts from alleles of both species. Total RNA extraction and cDNA synthesis were performed as previously described (Yazawa et al., 2010). To validate the histological observations, the reverse-transcription PCR (RT-PCR) for germ cell marker, *ddx4*; Sertoli cell marker, *gsdf*; Leydig cell marker, steroid 11-beta-hydroxylase (*cyp11b1*); and internal control, beta-actin (*actb*) was performed using cDNA obtained from the gonads of hybrid mackerel at 120-dph and 1-year-old. Moreover, to clarify whether hybrid mackerel have the potential to produce the 11-ketotestosterone (11-KT) that plays a pivotal role in spermatogenesis, RT-PCR for six steroidogenic enzyme genes required for conversion of cholesterol to 11-KT (cholesterol side-chain-cleavage enzyme, *cyp11a1*; 3 beta-

hydroxysteroid dehydrogenase / delta 5-delta 4 isomerase type I, *hsd3b1*; steroid 17 alpha-hydroxylase / C17,20 lyase, *cyp17a1*; hydroxysteroid 17-beta dehydrogenase 12, *hsd17b12*; *cyp11b1* and hydroxysteroid 11-beta dehydrogenase 2, *hsd11b2*) were performed using cDNA obtained from the testis of hybrid mackerel at 1-year-old. The PCR amplification was conducted with AmpliTaq Gold DNA polymerase; primer sets for each gene are listed in Table 1. To detect transcripts from alleles of both species, all primers were designed against regions that are completely conserved between chub and blue mackerel. The GenBank accession number of each gene of both chub and blue mackerel is listed in Table 2. Thermal cycling conditions were: 1 cycle of 95°C for 10 min, then 35 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, followed by a final elongation step at 72°C for 3 min. PCR products were electrophoresed on a 2.0% agarose gel.

Germ cell transplantation

Donor testicular cells were prepared from 3-year-old male Pacific bluefin tuna (body weight, approximately 40 kg) which were reared in net pens at Kushimoto, Wakayama Prefecture, Japan. Freshly isolated testes were minced with Weckel scissors and dissociated as previously described (Yazawa et al., 2013). To isolate spermatozoa and blood cells from whole testicular cell suspensions, density gradient centrifugation using a Percoll gradient (Percoll Plus; GE Healthcare, Princeton, NJ) was performed as previously described (Ichida

et al., 2019). To label donor testicular cells, PKH26 (Sigma-Aldrich, Inc., St. Louis, MO) staining was performed as described by Takeuchi et al. (2009). We previously revealed that chub mackerel larvae with a total length of 5.3-mm showed higher incorporation efficiency of transplanted germ cells relative to those with a total length of 4.2-mm or 6.9-mm (Yazawa et al., 2010). In this study, therefore, PKH26-labeled cells were transplanted into the peritoneal cavity of hybrid mackerel larvae at 10-dph with a total length of 5.8-mm. At least 10,000 cells were injected into each of the 170 recipients. Transplantation was performed as previously described (Yazawa et al., 2010). Genital ridges excised from five recipients were imaged under a fluorescent microscope (BX51N-34FL, Olympus) at 14 days post-transplantation to confirm the incorporation of PKH26-labeled donor germ cells. Incorporation rate of donor-derived germ cells in recipient genital ridges was calculated as [number of fish incorporating fluorescent cells in genital ridges at 14 days post-transplantation/number of fish observed x 100].

Statistical analysis

All data are represented as the mean ± standard error of the mean (SEM). A value of $p < 0.01$ was considered significant for all tests. A two-tailed Student's t-test was used to determine statistical differences in means of fertilization rates and hatching rates between hybrid mackerel and chub mackerel (F-test was performed to show that the variance of

populations were equal). Further, one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test was used to determine statistical significance in the mean GSI of 1- and 2-year-old hybrid mackerels and 1-year-old chub mackerels. Before conducting the ANOVA, the homogeneity of variances was determined with Bartlett's test. All analyses were carried out using GraphPad Prism version 5.0 (GraphPad).

Results

Production of hybrid mackerel

Early survival of hybrid mackerel is illustrated in Fig. 1A. Mean ± SEM values for fertilization and hatching rates of hybrid mackerel were 93.5% ± 2.3% and 87.4% ± 4.4% (n = 3), respectively. By comparison, mean fertilization and hatching rates of control blue mackerel were 96.7% ± 0.6% and 84.7% ± 3.6% (n = 3), respectively. No significant differences between these fertilization and hatching rates were found. Genomic DNA of hybrid mackerel larvae at 1-dph was subjected to multiplex PCR analysis that targeted a species-specific sequence of the ITS region in nDNA (Fig. 1B). As a result, both blue mackerel and chub mackerel specific fragments (200 bp and 125 bp, respectively) were amplified in all tested hybrid mackerel larvae (n = 10). Moreover, the PCR-RFLP targeted region of mtDNA indicated that hybrid mackerel larvae possessed mtDNA derived from blue mackerel; restriction patterns were identical to those of blue mackerel (Fig. 1C, n=10). Analyses of cellular DNA content revealed that hybrid mackerel showed the same peaks for DNA contents observed in diploid parental species and none exhibited signs of aneuploidy (Fig. 1D). Moreover, the external appearance of hybrid mackerel at 120dph was similar to parental species at the same age and showed no deformation. Hybrid fish grew normally (Fig. 1E).

Gonadal development of hybrid mackerel at 30-, 60- and 120-dph

To clarify the fertility of hybrid fish, gonadal development was compared with that of control chub mackerel (Fig. 2). At 30-dph, hybrid mackerel possessed sexually undifferentiated gonads with primordial germ cells (PGCs) that were enclosed by gonadal somatic cells (Fig. 2B), as also observed in controls (Fig. 2A). Control chub mackerel gonads at 60-dph differentiated into testis or ovary (Fig. 2C, D) and sperm duct and spermatogonia were observed in testis (Fig. 2C). The ovarian cavity and ovarian lamella were formed in control fish, and oogonia were observed (Fig. 2D). By contrast, all hybrid mackerel at 60-dph showed only testis-like gonads possessing sperm duct and spermatogonia (n = 10, Fig. 2E). In control at 120-dph, numbers of germ cells were drastically increased in both testis (Fig. 2F) and ovary (Fig. 2G). In ovaries, primary oocytes were observed during the perinucleolus stage (Fig. 2G). Hybrid mackerel at 120-dph showed only testis-like gonads as at 60-dph, and germ cells were not observed in these testis-like gonads (n = 10, Fig. 2H).

Gene expression analyses of germ cell-less gonads of hybrid mackerel at 120-dph

To further confirm depletion of germ cells, we investigated expression of the germ cell marker, *ddx4* mRNA, in hybrid mackerel gonads at 120-dph by ISH (Fig. 3A-F, A'-F'). *ddx4*-positive germ cells were detected in control blue mackerel testis (Fig. 3B, B'), whereas no *ddx4*-positive germ cells were detected in hybrid mackerel gonads (Fig. 3E, E'). Further,

transcripts for *ddx4* were not detectable using RT-PCR (Fig. 3G). *gsdf*-positive Sertoli cells were detected in both control blue mackerel and hybrid mackerel at nearly equal levels (Fig. 3C, C', F, F'). Thus, hybrid mackerel are devoid of germ cells, but not Sertoli cells. Expression of *gsdf* in the hybrid gonads was further confirmed by RT-PCR (Fig. 3G), as was the of Leydig cell marker, *cyp11b1* (Fig. 3G). These results are promising for germ cell transplantation because the presence of supporting cells and steroidogenic cells that can nurse donor-derived germ cells is a prerequisite for the successful colonization and development of these cells.

Histological analysis of hybrid mackerel gonads at 1- and 2-year-old

Male and female chub mackerel are reported to reach sexual maturity at one year in captivity (Ishibashi et al., 2007; Nyuji et al., 2014). We investigated 1- and 2-year-old hybrid mackerel for germ cell-less testis during spawning seasons. At one and two years after hatching, seven out of 11 (63.6 %) and eight out of 11 (72.7 %) hybrid mackerel possessed germ cell-less testes, respectively (Fig. 4A, G, M) and GSI of these germ cell-less testes was 0.04 ± 0.003% and 0.04 ± 0.007%, respectively both of which are significantly lower than testes of control chub mackerel at 1 year old (GSI; 6.75 ± 0.758% , Fig. 4E, K, Q) ($p > 0.01$). Three out of 11 (27.3%) 1-year-old and 2 out of 11 (18.2%) 2-year-old hybrid mackerel possessed spermiated testes and all stages of spermatogenic cells, i.e. spermatogonia to

sperm, was observed with HE staining (Fig. 4C, I, O), yet the GSI of these spermiated testes was 0.31 ± 0.12% and 1.9 ± 1.3%, respectively, were also significantly lower than testes of 1 year-old control chub mackerel ($p > 0.01$). Also, one out of 11 (9.1%) 1-year-old hybrid mackerel showed displayed an ovary with numerous oocytes (Fig. 4D, J, P). Moreover, one out of 11 (9.1%) 2-year-old hybrid mackerel possessed germ cell-less ovary-like gonad with an ovarian cavity and ovarian lamella (Fig. 4B, H, N). As mentioned above, although a 50:50 sex ratio is observed in wild chub mackerel, the sex ratio of male to female hybrid mackerel was 10:1 at both 1- and 2-years after hatching (Table 3). Thus, hybrid mackerel exhibit a strong male-biased sex ratio.

Gene expression analyses of testis of hybrid mackerel at 1-year-old

The expression of *ddx4*, *gsdf* and six steroidogenic enzyme genes required for conversion of cholesterol to 11-KT (*cyp11a1*, *hsd3b1*, *cyp17a1*, *hsd17b12*, *cyp11b1*, *hsd11b2*) in the testis of 1-year-old hybrid mackerel were investigated using RT-PCR (Fig. 5). The germ cell-less testis of 1-year-old hybrid mackerel were found to be *ddx4*-negative, *gsdf*-positive, and *cyp11b1*-positive. Thus, the expression pattern of each cell-type maker genes in the germ cell-less testis of 1-year-old fish was the same as those at 120-dph. Moreover, all tested steroidogenic enzyme genes were detected in both germ cell-less testis (*ddx4*-negative) and spermiated testis (*ddx4*-positive) of hybrid mackerel, as well as in the spermiated testis of

control blue and chub mackerel. These results suggested that the germ cell-less testis of hybrid mackerel have the ability to produce the 11-KT.

Transplantation of bluefin tuna testicular cells into the hybrid mackerel.

Bluefin tuna testicular cells labeled with PKH26 (Fig. 6A, B) were transplanted into the peritoneal cavity of hybrid larvae at 10-dph. At 24-dph, no fluorescence was observed in the gonads of non-transplanted fish (Fig. 6C-E), whereas numerous PKH26-labeled cells were incorporated in the gonad of transplanted recipient larvae (Fig. 6F-H). The frequency of occurrence of recipient larvae incorporating donor-derived bluefin tuna germ cells was 100% (n = 5). The mean number of donor-derived germ cells found in the hybrid mackerel recipients was 36.9 ± 8.4. Hybrid mackerel gonads thus possessed a capability to attract and incorporate donor germ cells of bluefin tuna at high efficiency.

Discussion

In this study, we succeeded in producing a hybrid mackerel (female blue mackerel × male chub mackerel) that lost germ cells in gonads after 120-dph. This loss was confirmed by lack of expression of a germ cell marker, *ddx4*. In contrast, gene expression of a Sertoli cell marker, *gsdf*, and a Leydig cell marker, *cyp11b1*, were clearly detected in the hybrid mackerel gonads. The hybrid mackerel thus shows germ cell-less sterility but retains supporting cells and steroidogenic cells both of which are essential for nursing transplanted exogenous germ cells. More importantly, bluefin tuna germ cells transplanted into the peritoneal cavity migrated to and were incorporated into hybrid mackerel gonads with high efficiency. The genital ridge of hybrid mackerel thus retains the capability to attract and incorporate xenogeneic bluefin tuna germ cells.

As a key regulator for guiding the migration of PGCs, the chemokine SDF-1 (stromal cell-derived factor 1, also called as CXCL12) secreted by the region where the gonad develops and its receptor CXCR4 (C-X-C chemokine receptor type 4) expressed in the PGCs have been identified in zebrafish (Doitsidou et al., 2002), medaka (Sasado et al., 2008), and kingfish (Fernández et al., 2015). We previously confirmed CXCR4 is also expressed in testicular germ cells (Hayashi et al., unpublished data). Therefore, hybrid mackerel SDF-1 may activate bluefin tuna CXCR4 signaling and guide the migration of transplanted bluefin tuna germ cells to the region where the gonad develops, even though they were isolated from

a different genus. In a previous study, migration of donor cells into recipient gonads was usually possible even if donor and recipient were genetically distant species (Yoshizaki and Yazawa, 2019; Goto and Saito, 2019). Notably, Saito et al. (2014) showed that transplanted sturgeon PGC was incorporated into the genital ridge of goldfish. These species are from different orders. These reports suggest that mechanisms of germ cell migration are conserved across fish species, and the present results are consistent with this hypothesis.

Incorporation rate (100.0%) found in the present inter-generic transplantation was higher than colonization rates (70.0%) observed in our previous inter-family transplantation using donor nibe croaker and recipient chub mackerel (Yazawa et al., 2010). Colonization frequency may be affected by the phylogenic distance between the donor and recipient species.

Overall, hybrid mackerel show significant promise as a recipient for production of bluefin tuna gametes by surrogate broodstock technology. Most hybrid mackerel are germ cell-less and hold advantages over sterile triploid fish. First, germ cell-less recipients produce only donor-derived gametes. Triploids produce small numbers of abnormal, aneuploid gametes in some species (Felip et al., 2001; Piferrer et al., 2009), leading to risks of producing both donor-derived gametes and dysfunctional endogenous gametes. Consequently, the efficiency of producing donor-derived offspring might be compromised undesirable fertilization with dysfunctional endogenous gametes. Since germ cell-less

recipients are completely devoid of endogenous germ cells, they will produce only donor-derived offspring. Second, since triploid gonads maintain a normal number of mitotic germ cells, endogenous cells are presumed to compete with the transplanted germ cells for germ cell niches. No such competitive endogenous germ cells exist in germ cell-less sterile fish. In rainbow trout, the donor-derived germ cells showed significantly higher colonization and proliferation efficiency in the gonads of germ cell-less recipients, produced by knockdown of *dnd* expression using antisense morpholino oligonucleotides (AMO), than those in the gonads of the control recipients (Yoshizaki et al., 2016). In contrast to *dnd*-AMO-treated recipients that show no germ cells during the transplantation period, hybrid mackerel possess endogenous germ cells until 60-dph. Therefore, the colonization efficiency of donor-derived germ cells might not be enhanced in the gonads of hybrid mackerel. On the other hand, since endogenous germ cells of most hybrid mackerels disappeared by 120-dph, the transplanted germ cells can occupy the germ cell niches effectively, meaning that their proliferation would be enhanced in the gonads of hybrid mackerel. Further, when testicular cells of sockeye salmon (*Oncorhynchus nerka*) are transplanted into triploid and *dnd*-knockdown rainbow trout, triploid trout produces donor-derived salmon sperm but not eggs, but the *dnd*-knockdown trout recipient successfully produces both donor-derived salmon sperms and eggs (Yoshizaki et al., unpublished data). In the present study, most hybrid mackerel possess germ cell-less gonads, making them promising recipients for production of xenogeneic

bluefin tuna gametes.

In various animal species, including fish, a cytological mechanism of hybrid sterility is meiotic arrest caused by failure to achieve synapsis between homologous chromosomes (Chandley et al., 1975; Shimizu et al., 1997; Sawamura et al., 2004; Bhattacharyya et al., 2013; Islam et al., 2013). Therefore, germ cell-less sterility in the hybrid mackerel is a rare case of hybrid sterility. Recently, Yoshikawa et al. (2018a) reported that a hybrid of female blue dram × male white croaker showed germ cell-less sterility, and this report is apparently to only one in the existing literature. The PGCs of hybrid larvae from this cross migrate and are incorporated into genital ridges normally but are unable to proliferate. Eventually, hybrid gonads become germ cell-less and sterile. In contrast, proliferation of PGCs in mackerel hybrid gonads was observed histologically at 60-dph, indicating that germ cell-less sterility of hybrid mackerel is caused by mechanisms different from mechanisms underlying sterility in blue dram × white croaker hybrids. Further study is required to investigate proliferation, differentiation, and apoptosis in germ cells found in the gonads of hybrid mackerel juveniles.

We revealed that hybrid mackerel show a strong male-biased sex ratio and individuals possessing ovary-like germ cell-less gonads rarely appeared. In zebrafish and medaka (*Oryzias latipes*), numbers of germ cells affect sex differentiation; that is, absence of germ cells leads to exclusive male development even in genetic females (Weidinger et al., 2003; Slanchev et al., 2005; Kurokawa et al., 2007; Li et al., 2017). This phenomenon also occurred

in hybrid mackerel possessing germ cell-less gonads. This male-biased sex ratio may become a potential disadvantage for production of donor-derived eggs. However, we have succeeded in producing feminized chub mackerel by oral administration of estradiol-17beta (Tani et al., unpublished data), the promising potential for production of female hybrid mackerel using this technique will be assessed in future work.

In order to nurse the spermatogenesis of transplanted exogenous germ cells, it is essential that the recipient has the ability to produce the androgens (11-KT) that play a pivotal role in spermatogenesis (Miura et al., 1991, Schulz and Miura, 2002). The RT-PCR analysis revealed that the steroidogenic enzyme genes, required for conversion of cholesterol to 11-KT, are expressed in the germ cell-less testis of 1-year-old hybrid mackerel. Since the germ cells depletion markedly affects the anatomical structure and frequency of occurrence of each cell type in testes, quantitative analyses of reproductive hormones in the germ cell-less testis were not performed. Certainly, however, the germ cell-less testis of hybrid mackerel do have the potential for androgen production. Future studies will be required to reveal whether transplanted hybrid mackerel recipients can nurse spermatogenesis of donor-derived germ cells.

The sterility of hybrid mackerel was not displayed in all individuals. The frequency of non-sterile fish was as low as 36.4% after 1 year, and 18.2% after 2 years, and 0% after the first four months (120-dph group). Since we produced these fish groups using different

broodstock, we could not rule out the possibility that the frequency of the sterile fish is affected by their genetic background. To produce a 100% sterile population, the precise mechanism of sterility caused by hybridization in mackerel will require better understanding.

In the present study, we showed biological characteristics of hybrid mackerel, with special emphasis on germ cell-less sterility. We also showed applicability of this hybrid for mass-production and its ability to harbor tuna germ cells. Thus, hybrid mackerel are promising recipient candidates as surrogate broodstock for bluefin tuna gamete production.

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Figure legends

Figure 1. Preparation of donor testicular cells. (a) A section of a donor chub mackerel testis stained with hematoxylin and eosin. Arrowheads indicate type-A spermatogonia. Dissociated testicular cells before (b) and after (c) density gradient centrifugation using a Percoll gradient. Arrowheads indicate predicted spermatogonia. Scale bars indicate 10 μm.

Figure 2. PCR-RFLP analysis of mtDNA for identification of genetic origin in sperm produced by hybrid recipients. (a) Schematic drawing for the design of germ cell transplantation experiments. CM; chub mackerel, BM; blue mackerel. (b) Amplified PCR fragments (505 bp) were undigested (U) and digested (D) with the restriction enzyme *Hae*III, which recognizes a species-specific sequence of chub mackerel DNA and produces two digested bands (262 and 243 bp). (c) Results of PCR-RFLP analysis of mtDNA. Lanes 1–35 are DNA from milt samples from individual male recipients. Lanes CM, BM, and Hyb are controls obtained using DNA extracted from the pectoral fin of CM, BM, and hybrid mackerel (BM×CM hybrid), respectively.

Figure 3. (a) Photograph of milt samples collected from transplanted hybrid recipients and wild-type chub mackerel (WT-CM). For each milt sample, a 1-μL sample was dropped onto a glass slide. (b) Production of donor-derived offspring from male recipients.

Fertilization (black bars) and hatching (white bars) rates of wild-type chub mackerel eggs inseminated with sperm of four hybrid recipients and WT-CM sperm. Data are presented as mean±SEM. Asterisk indicates statistically significant difference against WT-CM (*t*-test, $p<0.05$).

Figure 4. (a) Schematic drawing for the design of the progeny test of male recipients. Allele sizes at the SJ-30 locus of a donor chub mackerel, #1–4 male recipient fish, and wild type used as female parent are indicated by bold digits. (b) Typical morphology at 12 h post hatch of larva of wild-type chub mackerel (upper photo), F1 chub mackerel from donor-derived sperm (middle photo), and F1 hybrid mackerel from hybrid recipient-derived sperm (bottom photo). Bars=0.5 mm.

Figure 5. PCR-RFLP analysis of mtDNA for identification of genetic origin in unfertilized eggs obtained from female hybrid recipients. Amplified PCR fragments (505 bp) were undigested (U) and digested (D) with the restriction enzyme *Hae*III, which recognizes a species-specific sequence of chub mackerel DNA and produced two digested bands (262 and 243 bp). Results of 30 unfertilized eggs obtained from females #1 and #2 are shown in the upper and lower panels, respectively.

Figure 6. Schematic drawing for the design of the mating experiment. Allele sizes at the SJ-30 locus of a donor chub mackerel; recipient fish are indicated by bold digits. The origins of mtDNA in each fish are shown as CM, chub mackerel or BM, blue mackerel.

Fig. 1

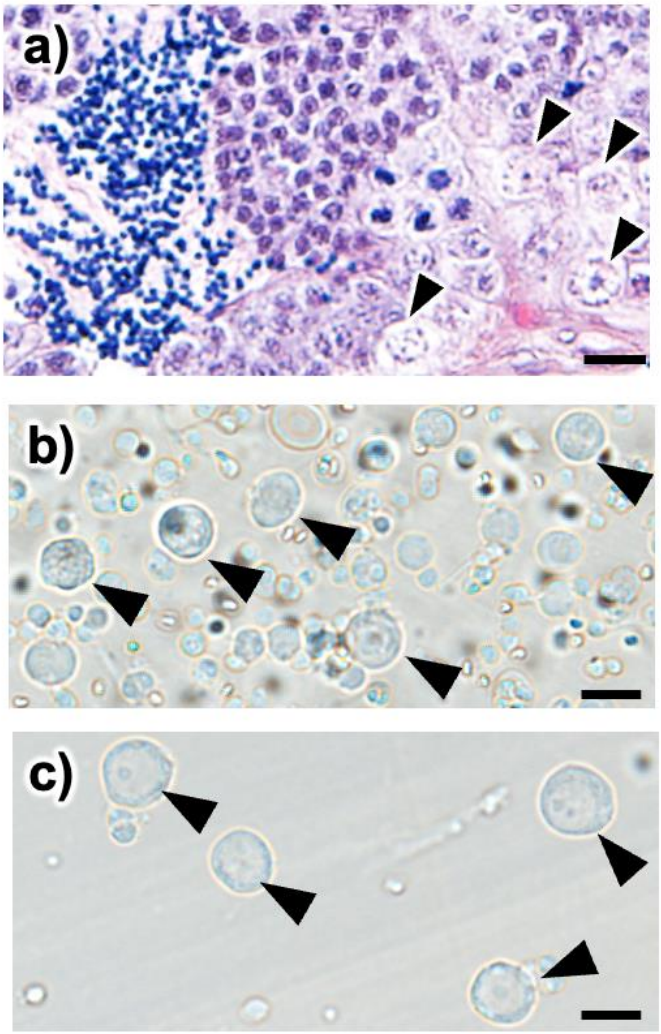


Fig. 2

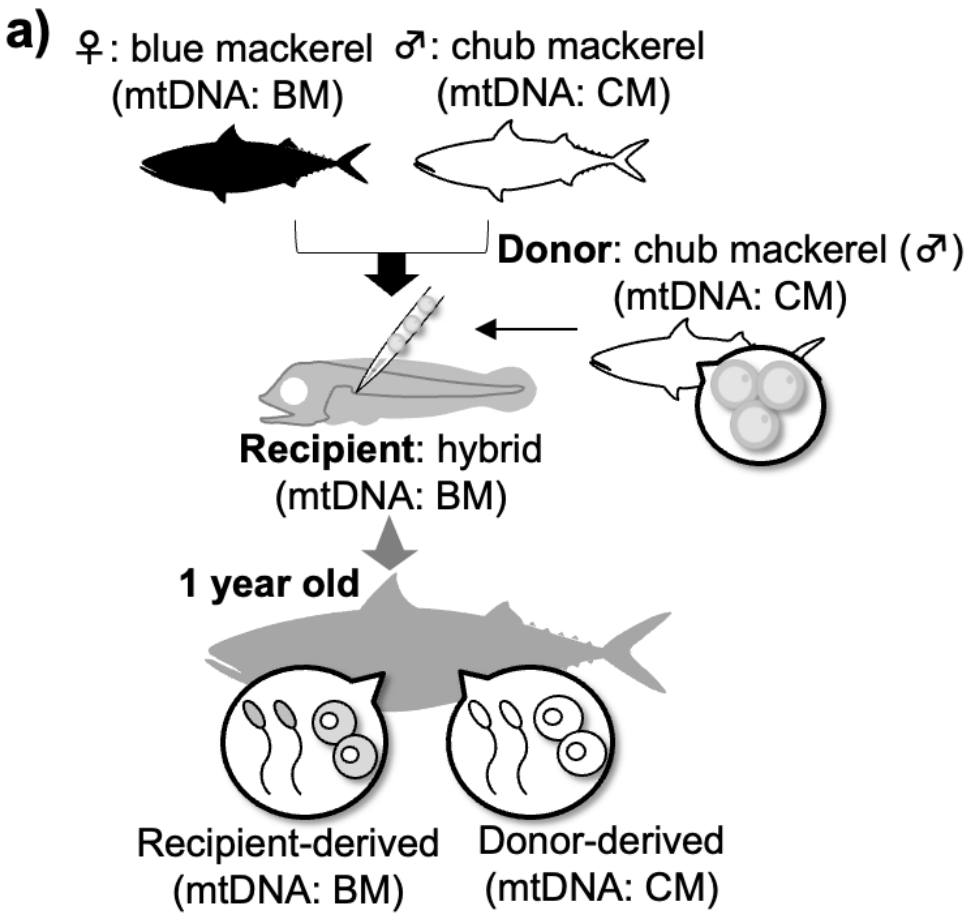

a)
♀: blue mackerel
(mtDNA: BM)
♂: chub mackerel
(mtDNA: CM)
Donor: chub mackerel (♂)
(mtDNA: CM)
Recipient: hybrid
(mtDNA: BM)
1 year old
Recipient-derived
(mtDNA: BM)
Donor-derived
(mtDNA: CM)

Fig. 2. contd.

b)

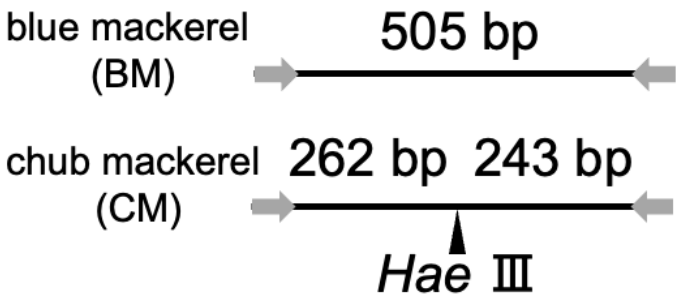


Fig. 2. contd.

c)

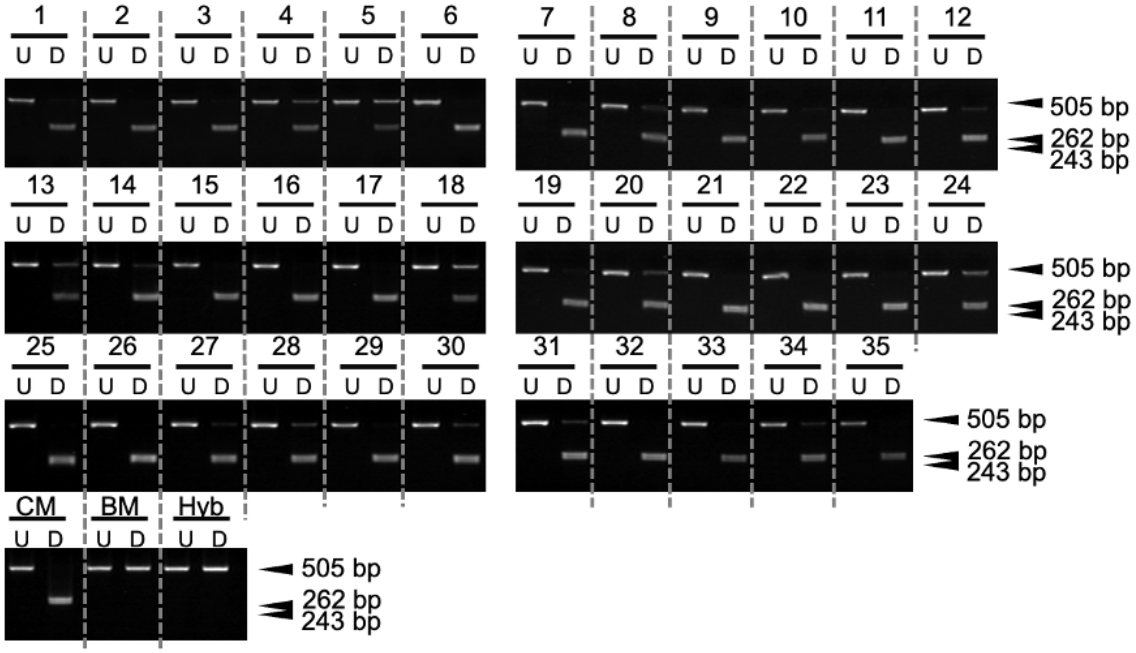

1 2 3 4 5 6
7 8 9 10 11 12
13 14 15 16 17 18
19 20 21 22 23 24
25 26 27 28 29 30
31 32 33 34 35
CM BM Hyb
U D
505 bp
262 bp
243 bp

Fig. 3

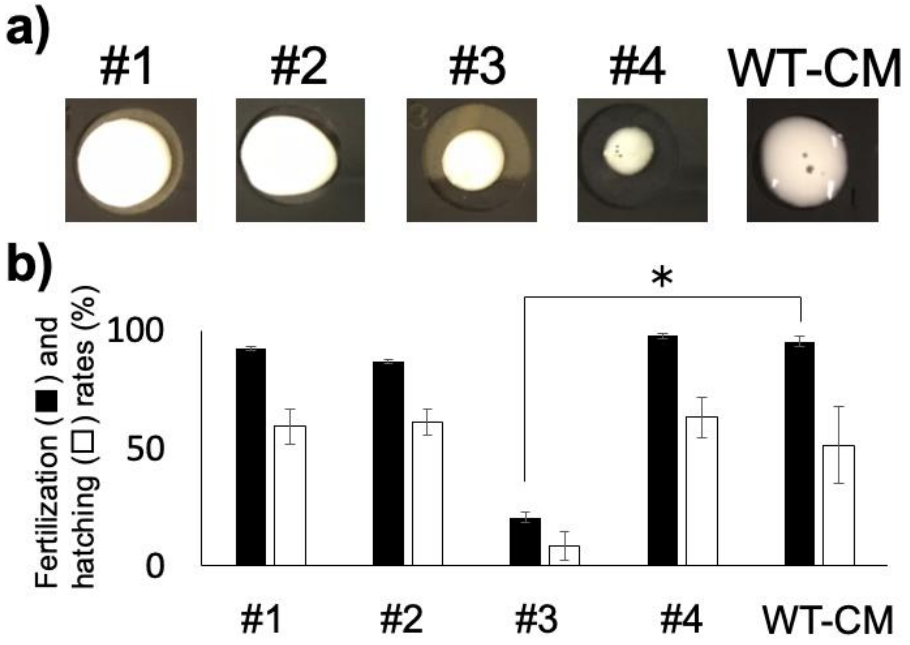

a)
#1
#2
#3
#4
WT-CM
b)
Fertilization (■) and hatching (□) rates (%)
100
50
0
*
#1
#2
#3
#4
WT-CM

Fig. 4

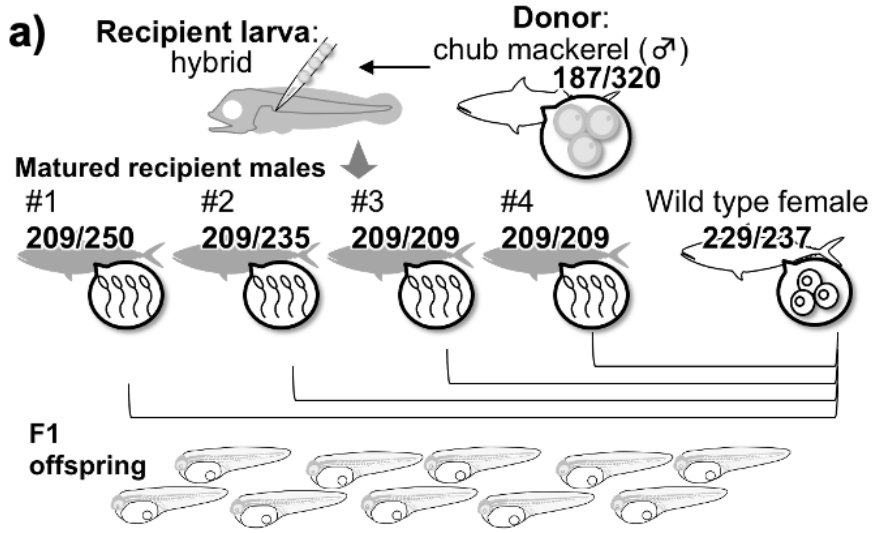

a)
Recipient larva:
hybrid
Donor:
chub mackerel (♂)
187/320
Matured recipient males
#1
209/250
#2
209/235
#3
209/209
#4
209/209
Wild type female
229/237
F1
offspring

Fig. 4

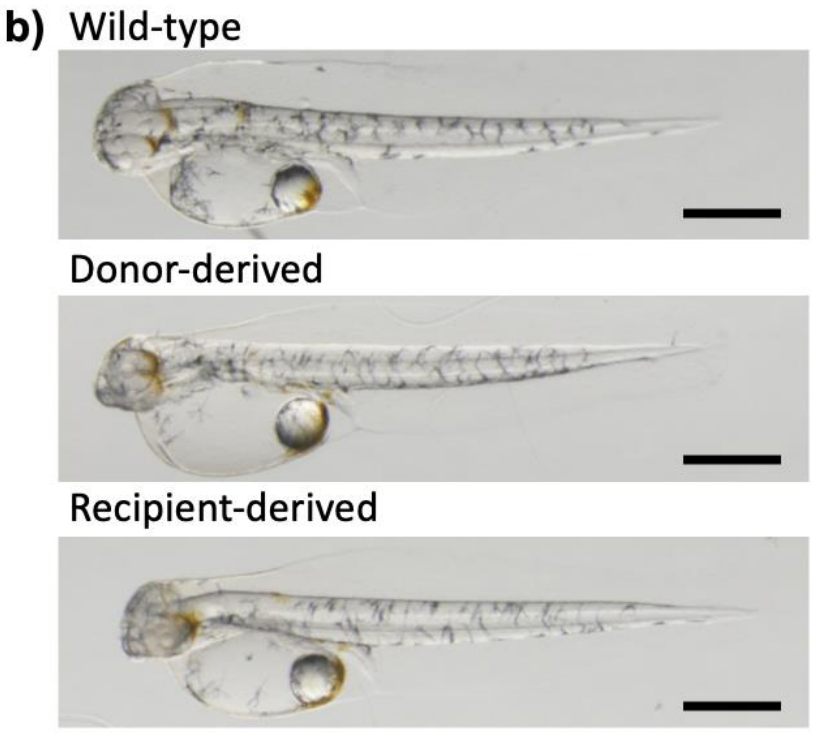


Fig. 5

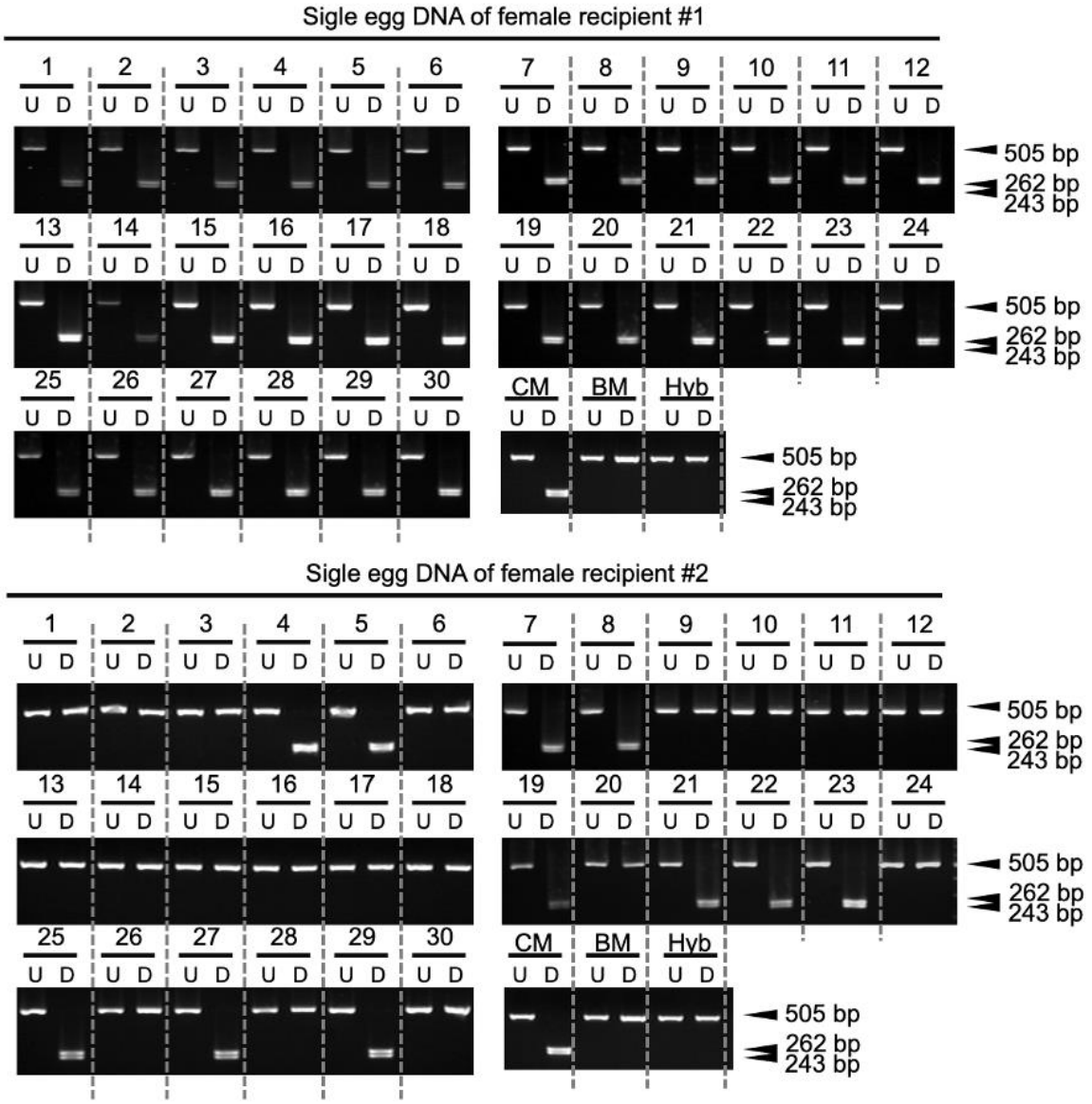
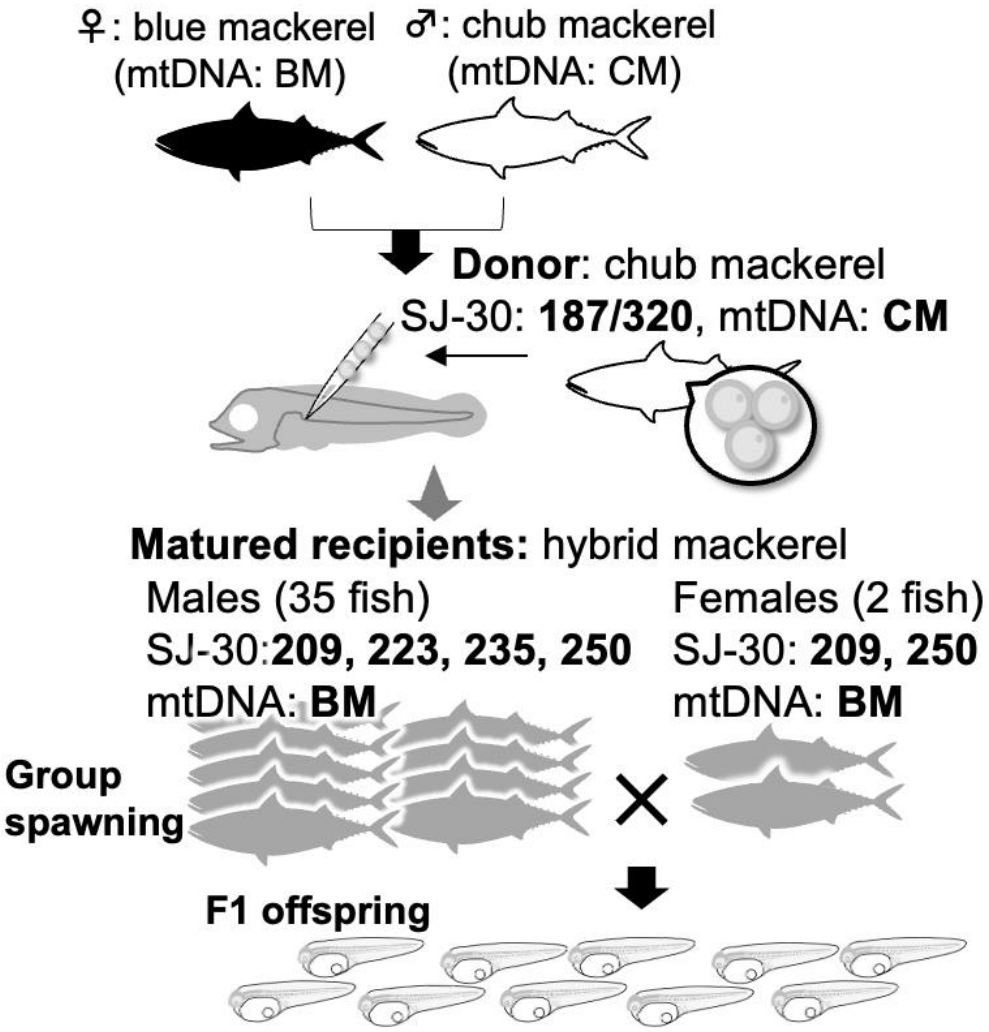

Sigle egg DNA of female recipient #1
1 2 3 4 5 6 7 8 9 10 11 12
U D U D U D U D U D U D U D U D U D U D U D U D
505 bp
262 bp
243 bp
13 14 15 16 17 18 19 20 21 22 23 24
U D U D U D U D U D U D U D U D U D U D U D U D
505 bp
262 bp
243 bp
25 26 27 28 29 30 CM BM Hyb
U D U D U D U D U D U D U D U D U D
505 bp
262 bp
243 bp
Sigle egg DNA of female recipient #2
1 2 3 4 5 6 7 8 9 10 11 12
U D U D U D U D U D U D U D U D U D U D U D U D
505 bp
262 bp
243 bp
13 14 15 16 17 18 19 20 21 22 23 24
U D U D U D U D U D U D U D U D U D U D U D U D
505 bp
262 bp
243 bp
25 26 27 28 29 30 CM BM Hyb
U D U D U D U D U D U D U D U D U D
505 bp
262 bp
243 bp

Fig. 6


♀: blue mackerel
(mtDNA: BM)
♂: chub mackerel
(mtDNA: CM)
Donor: chub mackerel
SJ-30: 187/320, mtDNA: CM
Matured recipients: hybrid mackerel
Males (35 fish)
SJ-30:209, 223, 235, 250
mtDNA: BM
Females (2 fish)
SJ-30: 209, 250
mtDNA: BM
Group spawning
F1 offspring

Tables

TABLE 1 Summary of transplanted hybrid mackerel recipients

	Survived (Total 48)		Matured (Total 37)	
Transplanted	Male	Female	Male	Female
500	42	6	35	2

TABLE 2 Microsatellite analysis in F1 larvae obtained by progeny test of male recipients

	F1 larvae				
Recipient # 1	187/229	187/237	320/229	320/237	
(209/250)	2	3	1	4	
Recipient # 2	187/229	187/237	320/229	320/237	
(209/235)	1	1	4	4	
Recipient # 3	187/229	187/237	320/229	320/237	
(209/209)	1	2	4	4	
Recipient # 4	187/229	187/237	320/229	320/237	209/237
(209/209)	3	2	0	4	1

Note: Donor chub mackerel 187/320; Wild type female 229/237. Upper column indicate genotype, and lower column indicate number of larvae.

TABLE 3 Summary of the spawning events of hybrid recipients

Days post administration	No. of floating eggs	No. of fertilized eggs	No. of hatched larvae
1	4,533	2,087	288
2	2,450	167	94
3	117	21	0
4	133	0	0

TABLE 4 Microsatellite and mtDNA PCR-RFLP analysis in F1 larvae obtained by mating experiment

Genotype	No. of larvae	mtDNA	Estimated origin of gametes	% in total 30 larvae
187/187	2	CM	Donor egg x Donor sperm	23.3%
187/320	4			
320/320	1			
209/187	4	CM	Donor egg x Recipient sperm	60.0%
250/187	6			
209/320	8			
209/209	2	BM	Recipient egg x Recipient sperm	16.7%
209/250	3			

Note: CM; chub mackerel, BM; blue mackerel

第 3 章

総括

総括

本研究により、ゴマサバ雌 x マサバ雄の組み合わせによるゴママサバ雑種宿主は移植したドナーマサバ生殖細胞に由来する卵および精子を生産可能であることが明らかとなった。これら移植宿主の一部の個体は、自身の配偶子を生産せず、ドナー由来の配偶子のみを生産していることが明らかになった。これにより、生殖細胞欠損型の不妊であるゴママサバ雑種が、実際にドナー由来の配偶子を効率よく生産できる優れた宿主であることが示された。また、ドナーのマサバ 1 尾に由来する生殖細胞を移植したゴママサバ宿主 37 尾を成熟まで飼育し、これら雑種宿主同士の自然産卵により実際にドナー由来の次世代を生産することに成功した。今後、ゴママサバ雑種における不妊化率の安定化や性比コントロールなどの課題を解決する必要があるものの、本技法が選抜個体喪失リスクの低いマサバ育種の遂行に実装できる可能性を示した。さらに、凍結保存したドナー精巣より得られた生殖細胞を雑種宿主に移植することで、ドナー由来の精子を生産可能であることも実証した。今後、凍結保存した生殖細胞に由来する卵も生産できることを確認する必要があるが、本技術を用いることで、将来的に確立されるであろうサバ類の優良系統において生体の維持が必須で無くなり、低コスト、省労力で、かつ半永久的に遺伝子資源を保存することが可能となると期待される。これにより、効率的かつ持続的なサバ類の養殖の実現に向けて、大きく貢献可能であると考えられる。

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