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PCR-Based Evaluation of Aquaculture Status in *Takifugu rubripes* Products

Soo Min Lee†, Jeongeun Park†, Hyunjung Kang, Minjeong Kim, Jieun Kim, Yunjeong Ji, Tae Sun Kang*

College of Science and Convergence Technology,
Department of Food Science and Technology, Seoul Women's University, Seoul, Korea

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ABSTRACT - Pufferfish are a valuable seafood resource in East Asia but they pose food safety risks due to tetrodotoxin (TTX) being present in wild populations. The pufferfish *Takifugu rubripes*, which are widely consumed in Korea, Japan, and China, are primarily cultivated under aquaculture conditions to produce TTX-free products, highlighting the need to distinguish them from wild individuals. In the present study, 100 commercial *T. rubripes* products from domestic markets were assessed using a PCR-based method targeting six genetic deletion markers previously validated for aquaculture status discrimination. The cultured products exhibited complete amplification across all of the markers, whereas the wild products showed variable patterns (0-5 amplified markers). These findings confirm that PCR-based amplification profiles represent a robust tool for verifying labeling accuracy, mitigating TTX-associated risks, and supporting the regulatory oversight of pufferfish products.

Key words: *Takifugu rubripes*, Genetic markers, PCR authentication, Molecular food safety

Recent dietary trends indicate increasing seafood consumption, driven by health consciousness and interest in sustainable diets. In Korea, per capita seafood consumption reached 65.6 kg in 2023, corresponding to an average annual growth rate of 2.2%[1]. Compared with meat products, seafood imposes a lower environmental burden in terms of climate change[2] and provides abundant omega-3 fatty acids and essential minerals, supporting both affordability and global demand[3]. Among seafood resources, pufferfish represent valuable fishery products and culinary ingredients in Asia. However, consumption of improperly processed or mislabeled pufferfish has been associated with tetrodotoxin (TTX) poisoning[4]. Despite the potent toxicity of TTX, several *Takifugu* species, including *T. rubripes* (commonly known as the tiger puffer), are highly prized in East Asian cuisine for their flavor and nutritional quality, making them commercially valuable in China, Japan, and Korea[5].

Within this genus, *T. rubripes* is extensively cultivated under controlled aquaculture conditions to ensure the production of TTX-free products. Cultured *T. rubripes* is preferred over wild individuals for several reasons. First, cultured fish are generally free of TTX, as they are maintained on TTX-free diets, whereas wild fish accumulate TTX through toxin-containing prey[6]. Consequently, cultured products pose a substantially lower risk of TTX-induced food poisoning. Second, cultured *T. rubripes* generally attain larger body sizes due to selective breeding and optimized rearing conditions. Additionally, cultured fish can be distributed live, further enhancing marketability[7].

Traditionally, pufferfish species are classified based on morphological and meristic traits such as skull shape, dermal spines, and dorsal fin ray counts[8]. However, high morphological similarity among many species complicates accurate identification. In particular, distinguishing closely related species such as *T. rubripes*, *T. chinensis*, and *T. pseudommus* remains challenging due to both morphological and genetic similarities. Although Korea regulates the trade of pufferfish under the Food Code (Article 2)[9] for 21 designated domestic and imported species, these regulations do not distinguish between closely related species or between wild and cultured *T. rubripes*. Moreover, conventional morphological methods cannot reliably differentiate cultured from wild *T. rubripes*.

To address this limitation, we recently performed a comparative genomic analysis between wild *T. pseudommus* and the reference *T. rubripes* genome (fTakRub1.2), identifying 226 large deletions (>1 kb) in the *T. pseudommus* genome[10].

†These authors contributed equally to this work.
*Correspondence to: Tae Sun Kang, College of Science and Convergence Technology, Department of Food Science and Technology, Seoul Women's University, Seoul 01797, Korea.
Tel: +82-2-970-5637, Fax: +82-2-970-5977
E-mail: missa1976@swu.ac.kr

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From these, six deletion markers (Ch1-5, Ch9-1, Ch18-9, Ch20-1, Ch20-4, and Ch20-13) were validated as reliable discriminators between cultured and wild *T. rubripes*[11]. Building on these findings, the present study evaluated the aquaculture status of 100 commercial *T. rubripes* products obtained from online and offline markets in Korea, applying a PCR-based method utilizing the six validated deletion markers to determine their origin (cultured vs. wild).

Materials and Methods

Sample collection

Six live farmed *T. rubripes* individuals, used as reference samples, were obtained in August 2024 from Aquatopia International (Tongyeong, Korea), the sole domestic company engaged in artificial fertilization, juvenile production, and distribution for *T. rubripes* aquaculture. In Korea, the sale of cultured pufferfish requires government-issued certification confirming TTX-free status. Between June 2023 and January 2024, a total of 100 commercial *T. rubripes* products were collected from a local pufferfish restaurant and online markets (Table 1). As reported previously, *T. rubripes, T. chinensis*, and *T. pseudommus* exhibit high morphological similarity and cannot be distinguished based on whole mitochondrial DNA, which strongly implies that they represent a single species within the same genetic pool[10]. Consequently, these species are marketed interchangeably as “Jajubok” and/or “Chambok” in Korean markets without taxonomic distinction. The species identities of the commercial products were verified in prior studies using DNA barcoding and morphological analyses[11], confirming that all samples belonged to one of the three species: *T. rubripes, T. chinensis*, or *T. pseudommus*.

Table 1. Sample information and conventional PCR results

Sample	Type[a]	Status[a]	Source	Primer sets for six large deletions						No. of amplicon
				Ch1-5	Ch9-1	Ch18-9	Ch20-1	Ch20-4	20-13	
S1				+	-	-	+	-	-	2
S2				-	+	+	+	+	+	5
S3				-	-	-	-	-	-	0
S4				-	-	-	-	-	-	0
S5				+	-	+	+	+	-	4
S6				-	-	+	+	-	-	2
S7				+	-	+	+	-	-	3
S8	Frozen	Wild	Online	-	-	-	+	+	-	2
S9				-	-	+	+	-	-	2
S10				-	-	+	+	-	-	2
S11				+	-	+	+	-	+	4
S12				-	-	+	-	-	-	1
S13				-	+	+	-	-	+	3
S14				-	-	-	+	-	+	2
S15				-	-	-	-	-	-	0
S16	Live	Farmed	Restaurant	+	+	+	+	+	+	6
S17				+	+	-	-	+	-	3
S18				+	+	-	-	-	-	2
S19				-	+	+	-	-	-	2
S20				+	-	-	+	-	-	2
S21				-	-	-	-	-	-	0
S22	Frozen	Wild	Online	+	-	-	-	+	-	2
S23				-	-	-	+	-	-	1
S24				-	-	-	+	-	-	1
S25				+	-	-	+	-	-	2
S26				-	-	-	+	-	-	1
S27				-	-	-	+	+	-	2

Table 1. (Continued) Sample information and conventional PCR results

Sample	Type[a]	Status[a]	Source	Primer sets for six large deletions						No. of amplicon
				Ch1-5	Ch9-1	Ch18-9	Ch20-1	Ch20-4	20-13	
S28				-	-	-	+	-	-	1
S29				+	-	-	-	-	-	1
S30				-	-	-	+	-	-	1
S31				-	-	-	+	-	-	1
S32				-	+	-	-	-	-	1
S33				-	-	+	-	-	-	1
S34				-	-	+	+	-	-	2
S35				-	-	+	+	+	-	3
S36				-	-	-	+	-	+	2
S37				+	-	-	+	+	-	3
S38				-	-	+	+	-	+	3
S39				-	-	-	+	-	-	1
S40				-	-	-	+	-	+	2
S41				-	-	-	+	-	-	1
S42				-	-	+	+	-	-	2
S43				-	+	+	+	-	-	3
S44				-	+	-	+	-	+	3
S45				+	-	-	+	-	-	2
S46				-	+	+	+	-	-	3
S47				-	-	+	+	-	+	3
S48				-	-	+	-	-	-	1
S49	Frozen	Wild	Online	+	-	-	+	-	-	2
S50				-	-	+	+	+	+	4
S51				-	+	-	+	-	+	3
S52				+	-	-	-	-	-	1
S53				-	-	-	+	-	-	1
S54				-	-	-	-	-	-	0
S55				-	-	-	+	-	-	1
S56				+	-	+	-	+	+	4
S57				+	-	-	+	+	+	4
S58				+	+	-	+	-	+	4
S59				-	-	+	-	-	-	1
S60				-	-	-	+	-	-	1
S61				-	-	-	-	-	+	1
S62				-	-	-	+	-	+	2
S63				-	-	+	+	-	+	3
S64				-	-	-	+	-	+	2
S65				+	-	-	+	-	-	2
S66				-	+	-	+	-	-	2
S67				-	-	+	+	-	-	2
S68				-	-	+	+	-	-	2
S69				-	-	-	-	+	-	1
S70				-	-	-	+	-	-	1
S71				-	-	+	-	+	-	1

Table 1. (Continued) Sample information and conventional PCR results

Sample	Type[a]	Status[a]	Source	Primer sets for six large deletions						No. of amplicon
				Ch1-5	Ch9-1	Ch18-9	Ch20-1	Ch20-4	20-13	
S72				-	-	-	+	-	-	1
S73				-	+	+	+	-	-	3
S74				-	-	-	+	+	-	2
S75				-	-	-	-	-	-	0
S76				-	-	-	-	-	-	0
S77				-	-	-	-	+	-	1
S78				-	-	+	+	+	+	4
S79				-	-	-	+	-	-	1
S80				-	-	-	-	-	-	0
S81				-	-	-	+	-	-	1
S82				-	-	+	+	-	-	2
S83				-	-	-	-	-	-	0
S84				-	-	+	+	-	-	2
S85				-	-	-	-	-	-	0
S86	Frozen	Wild	Online	-	-	+	-	+	-	2
S87				-	-	-	+	-	-	1
S88				-	-	-	+	-	-	1
S89				-	-	-	-	-	-	0
S90				+	-	+	+	+	-	4
S91				-	-	-	-	-	-	0
S92				-	+	+	+	-	-	3
S93				-	-	-	-	-	+	1
S94				-	-	+	+	-	+	3
S95				-	-	+	+	-	-	2
S96				-	+	-	+	-	-	2
S97				-	-	+	+	-	-	2
S98				-	-	+	-	-	-	1
S99				-	-	+	-	-	-	1
S100				-	-	-	+	-	-	1

[a)] Status was declared in the labels of commercial products.
+: Complete amplification, -: Non-amplification.

DNA extraction and quantification

For DNA extraction from processed products containing salt and seasonings, samples were thoroughly washed with distilled water and then preserved in 95% ethanol, as previously described. DNA was extracted from 30 mg of the edible portion of each sample using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted DNA was quantified with a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The purity of the extract was assessed by calculating the ratio of absorbance at 260 nm to that at 280 nm.

PCR amplification of six deletion markers

Specific primer sets used for the amplification of six deletion regions (Ch1-5, Ch9-1, Ch18-9, Ch20-1, Ch20-4, and Ch20-13) are listed in Table 2. These primer sets had been previously validated, demonstrating a sensitivity of 0.1 ng/μL and consistent amplification specificity in aquaculture-derived *T. rubripes*[11]. Conventional PCR was

Table 2. Oligonucleotide primer sequences used in this study

Name	Sequence (5'→3')	Size (bp)	Reference
Takifugu COI F	ATCATGATTGGAGGCTTTGG	124	7)
Takifugu COI R	CGAGCAGAAGGAGGAAGGAT		
Ch1-5 F	GGTGCACCGTTAGAAGGACA	426	7,11)
Ch1-5 R	TGGGGCCAGTCATGGTTTTT		
Ch9-1 F	ACATACAGACACACCGCGAA	402	7,11)
Ch9-1 R	CACTCGTGACAGTGAGGACC		
Ch18-9 F	GACGTCAGTTGAGTCCCTGG	419	7,11)
Ch18-9 R	ACCATCACGAGCACCTTCTG		
Ch20-1 F	TGCATTTCCGTCTCATGGCT	490	7,11)
Ch20-1 R	CCGCGATAAACGAGGGATCA		
Ch20-4 F	ATTGTTCAAGCCCAGGTCCG	455	7,11)
Ch20-4 R	TGTCCCTACTGTGGTGCAAT		
Ch20-13 F	GTTACGAGTGGCAGACACGA	401	7,11)
Ch20-13 R	GTCTCTCAGCTGATGCCTGT		

performed in a total reaction volume of 20 μL, consisting of 10 ng of template DNA, 0.5 μM of each primer, 10 μL of PCR Premix (EmeraldAmp PCR Master Mix, Takara, Japan), and sterile distilled water. The thermal cycling conditions employed were as follows: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 60°C for 20 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 3 minutes, using a thermal cycler 2720 (Applied Biosystems, Foster City, CA, USA). The *Takifugu* COI universal primer set was applied in both positive amplification control (PAC) and negative amplification control reactions (Table 2), with a non-template reaction serving as the negative control. PCR products were separated on 1% agarose gels stained with RedSafe (iNtRON, Seongnam, Korea), and fragment sizes were determined using a 1 kb DNA ladder (Bioneer, Daejeon, Korea).

Results and Discussion

DNA extraction and PCR amplification

The quantity and purity of DNA extracts from commercial products represent the first key step for successful PCR amplification[12]. In this study, DNA was extracted from a total of 100 *T. rubripes* products and the average concentration and purity of DNA extracts were 50.2 ± 9.72 ng/μL and 2.00 ± 0.12, respectively. The integrity of DNA extracts was tested using a PAC that can guide whether PCR-amplifiable DNA is present in the samples. This PAC has been successfully used as a control system that assesses amplification, inhibition, and false negative results of PCR-based methods[12]. In our recent study, universal *Takifugu* COI primers were designed to specifically target *T. rubripes*, *T. pseudommus*, and *T. chinensis*[7], which was used as the PAC. As shown in Fig. 1, universal *Takifugu* COI primers produced a conserved 124-bp fragment in all samples, indicating that the DNA extracts were suitable for further amplification of the six deletion markers.

PCR amplification of six deletions in *Takifugu rubripes* products

DNA extracted from six live farmed *T. rubripes* were subjected to PCR amplification using six deletion marker primers to validate their performance prior to assessing the labeling compliance of commercial samples. As expected, all six deletion primer sets, as well as the PAC control, yielded complete amplification profiles, consistently producing amplicons of the anticipated sizes (124-490 bp) from live farmed *T. rubripes* (Fig. 2). It is generally reported that cultured aquatic stocks are genetically distinct from their counterparts due to physically divided habitats, variations in feed, artificial reproduction, and a strong founder effect[10]. Our recent study strongly demonstrated that *T. rubripes*, *T. chinensis*, and *T. pseudommus* constitute a single species within the same genetic pool; however, cultured *T. rubripes* showed highly significant differentiation from its wild counterpart groups[10]. This result aligns with our previous study[10], which was primarily attributed to the low genetic diversity of cultured *T. rubripes*.

Subsequently, DNA from 100 commercial samples was analyzed with the same six primer sets. As summarized in

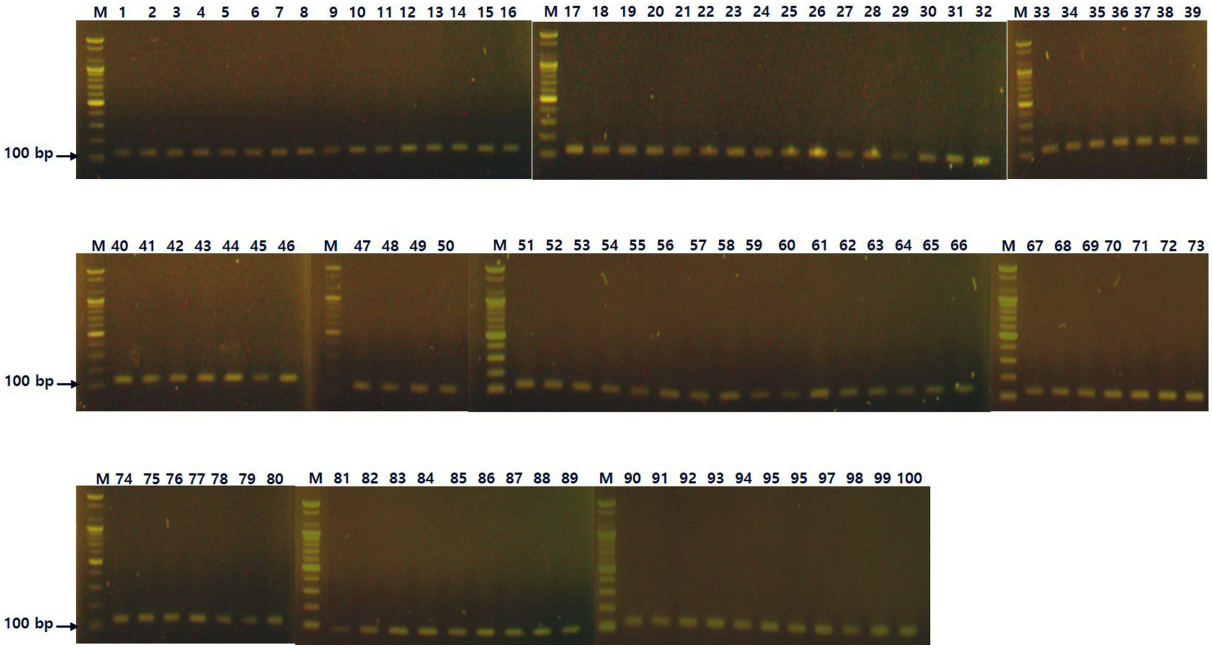

Fig. 1. DNA integrity results using universal *Takifugu* COI primers. M: 100 bp DNA ladder; 1-100: Commercial samples listed in Table 1.

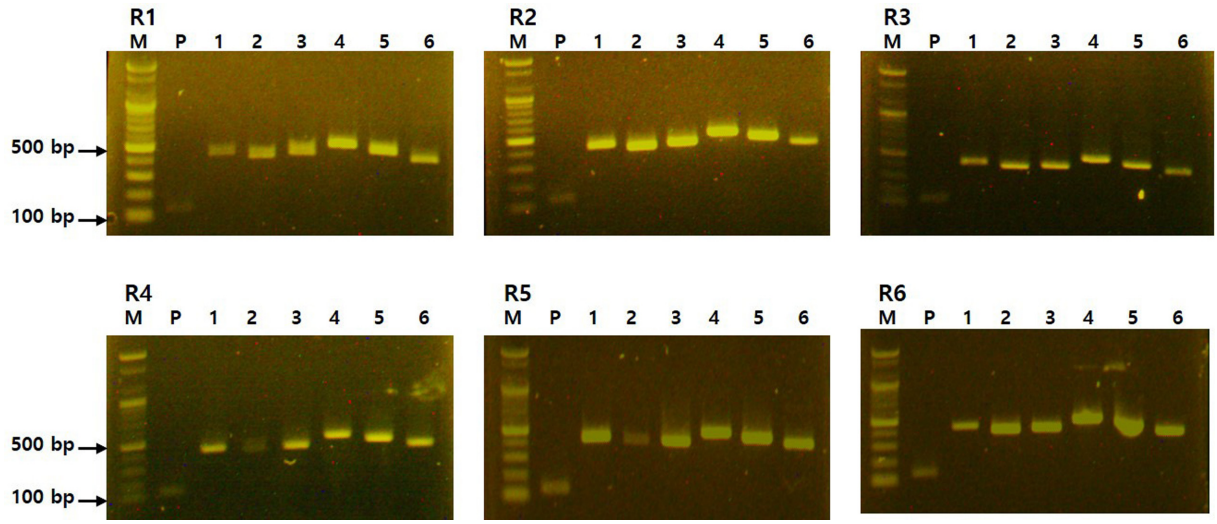

Fig. 2. PCR amplification patterns of six farmed *Takifugu rubripes*. R1-R6: Six farmed *Takifugu rubripes* samples. M: 100 bp DNA ladder; lane 1: Positive amplification control; lanes 2-7: Ch1-5, Ch9-1, Ch18-9, Ch20-1, Ch20-4, and Ch20-13 deletions, respectively.

Table 1 and Supplementary Fig. 1, these primer sets generated variable amplification patterns across all commercial *T. rubripes* samples, with the exception of sample S16, which displayed a complete amplification profile. This finding strongly suggests that S16 originated from farmed *T. rubripes*. In contrast, the remaining 99 samples exhibited incomplete and random amplification patterns. Specifically, 0, 1, 2, 3, 4, and 5 amplicons were detected in 12, 32, 31, 15, 8, and 1 samples, respectively, indicating that these samples most likely represented wild *T. rubripes*. Notably, the deletion markers amplified within groups producing the same number of amplicons were not identical. For example, both S12 and S23 each yielded a single amplicon; however, the Ch18-9 deletion was amplified in S12, whereas the Ch20-1 deletion was amplified in S23. These inconsistent and random amplification patterns are mostly attributed to higher genetic diversity caused by random mating, compared to its cultured

counterparts. Therefore, the six deletions based on genetic diversity can successfully discriminate wild and aquacultured *T. rubripes*.

Consistency between aquaculture status and labeling

To assess labeling compliance, the aquaculture status indicated on product labels was compared with PCR amplification patterns. As summarized in Table 1, PCR amplification patterns for all commercial products were entirely consistent with the aquaculture status indicated on labels. This finding contrasts with our previous monitoring study, which reported a high mislabeling rate (70%) in pufferfish products between the raw materials used and the information on their labels[13]. Of the 100 commercial *T. rubripes* products examined, 99 were processed pufferfish products with heads and intestines removed, purchased online, and labeled as wild-caught from Korea. Sample S16, which showed complete amplification across all six deletion markers, was a live specimen obtained from a local pufferfish restaurant and labeled as farmed from China. In our previous study, *Lagocephalus cheesemanii*, a relatively inexpensive species, was used in approximately 90% of pufferfish products imported from China[13]. Therefore, due to price differences, frozen *T. rubripes* products are generally not misidentified as wild *T. rubripes* from China or domestic sources. However, wild live *T. rubripes* are typically sold at higher prices than farmed individuals and require regular monitoring.

Our PCR-based method provides a rapid and accurate approach for determining the aquaculture status of *T. rubripes* products. Furthermore, this genetic approach could potentially be applied to distinguish toxic from non-toxic *T. rubripes*, because cultured *Takifugu* species are generally free of TTX due to their TTX-free diets[6].

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국문요약

복어는 동아시아 요리의 고급 원료로 상업적 가치가 높은 수산자원이나, 야생 복어의 테트로도톡신은 치명적인 식중독 사건을 꾸준히 유발하고 있다. 자주복은 한국, 일본, 중국에서 인기 있는 복어 종으로, 테트로도톡신이 없는 복어 생산을 위해 양식되는 주된 어종이다. 따라서 양식 자주복과 자연산 자주복의 구별은 식품 안전과 규제 측면에서 매우 중요하다. 본 연구에서는 국내 온라인 및 오프라인 시장에서 판매되는 100개의 자주복 제품을 대상으로 '양식 및 자연산' 여부를 PCR 기반 방법을 이용하여 확인하였으며, PCR 결과를 제품의 표시사항 정보와 비교하였다. PCR 분석에는 자연산 자주복과 양식 자주복의 유전적 다양성 차이를 보이는 6개의 마커를 이용하였다. PCR 분석 결과 모든 양식 자주복 제품에서는 6개 마커에서 모두 완전한 증폭 패턴을 보였으나, 자연산 자주복 제품에서는 0-5개 마커에서 만 무작위 증폭 패턴을 나타냈다. 따라서 6개 유전 마커의 증폭 패턴을 이용한 분석법은 향후 자주복 제품의 허위표시 모니터링 및 테트로도톡신 유무의 신속 검사에 널리 활용될 수 있을 것이다. 또한 본 연구 결과는 현행 복어 종 판별을 보완할 수 있는 기초자료를 제공해 줄 수 있다.

Conflict of interests

The authors declare no potential conflict of interest.

ORCID

Soo Min Lee https://orcid.org/0009-0009-8196-1308
Jeongeun Park https://orcid.org/0009-0005-2261-688X
Hyunjung Kang https://orcid.org/0009-0007-6734-7147
Minjeong Kim https://orcid.org/0009-0004-6673-8099
Jieun Kim https://orcid.org/0009-0003-7544-6234
Yunjeong Ji https://orcid.org/0009-0004-4656-1353
Tae Sun Kang https://orcid.org/0000-0001-5939-6247

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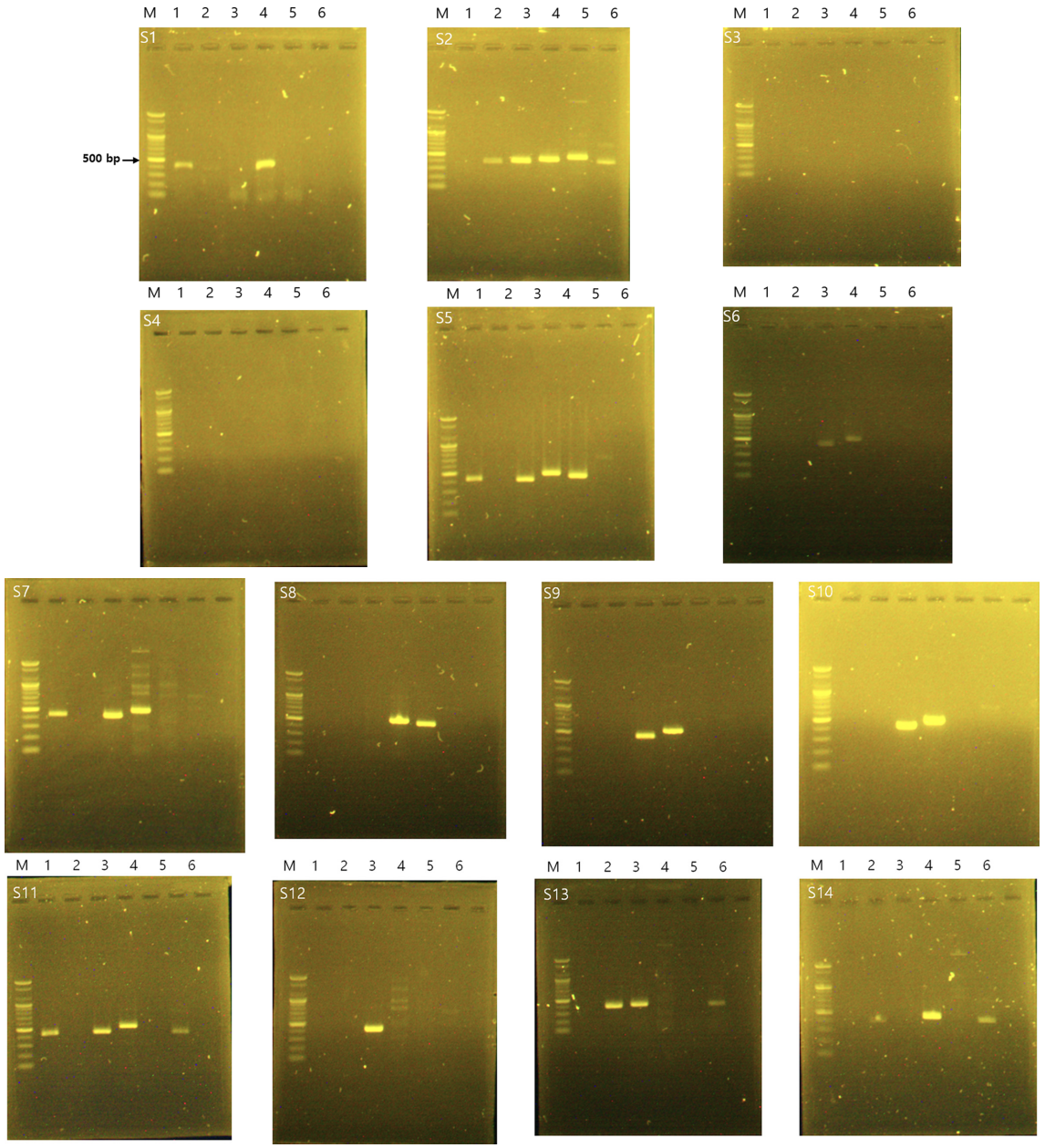
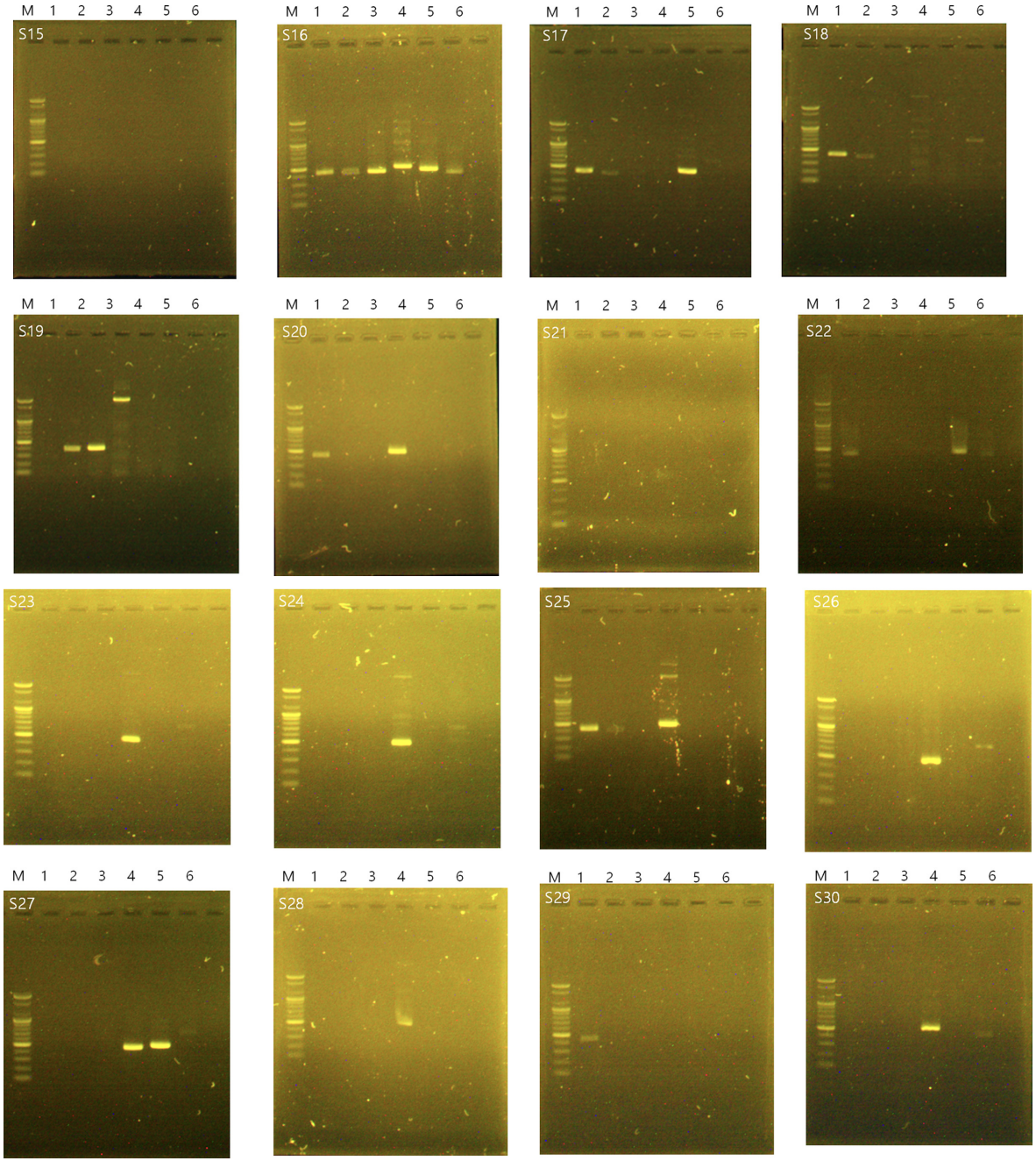
Supplementary materials

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S23
S24
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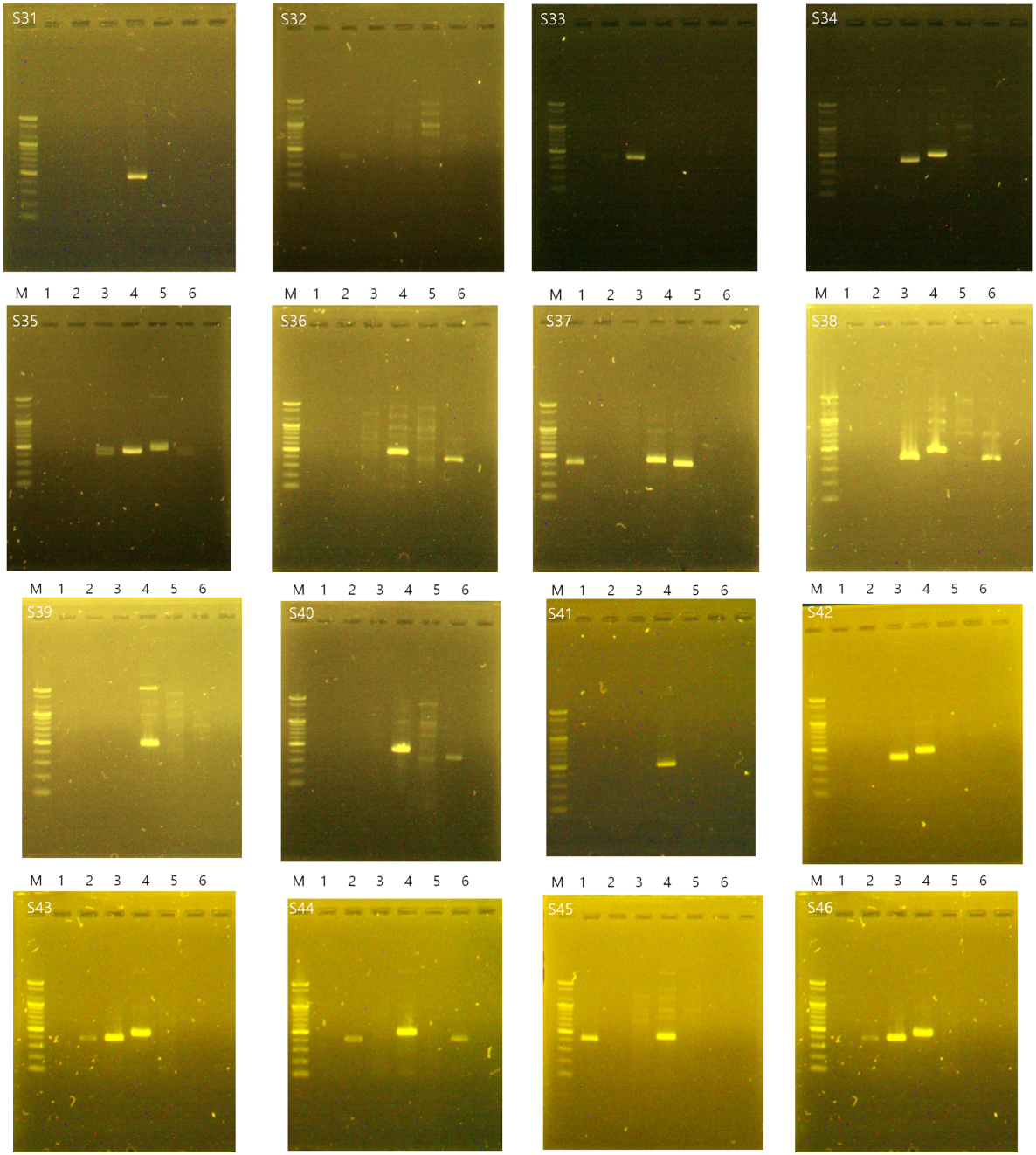
S30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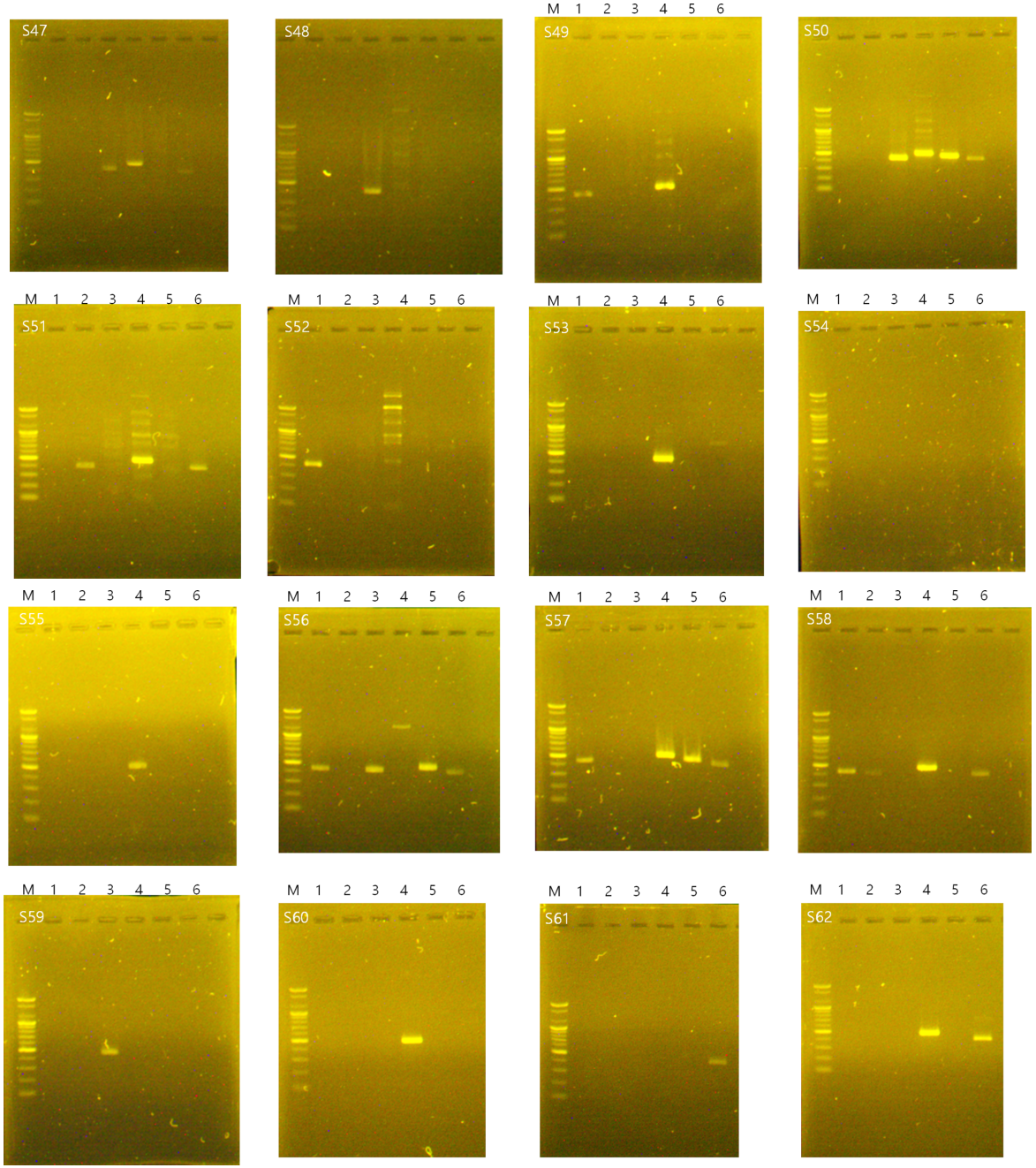
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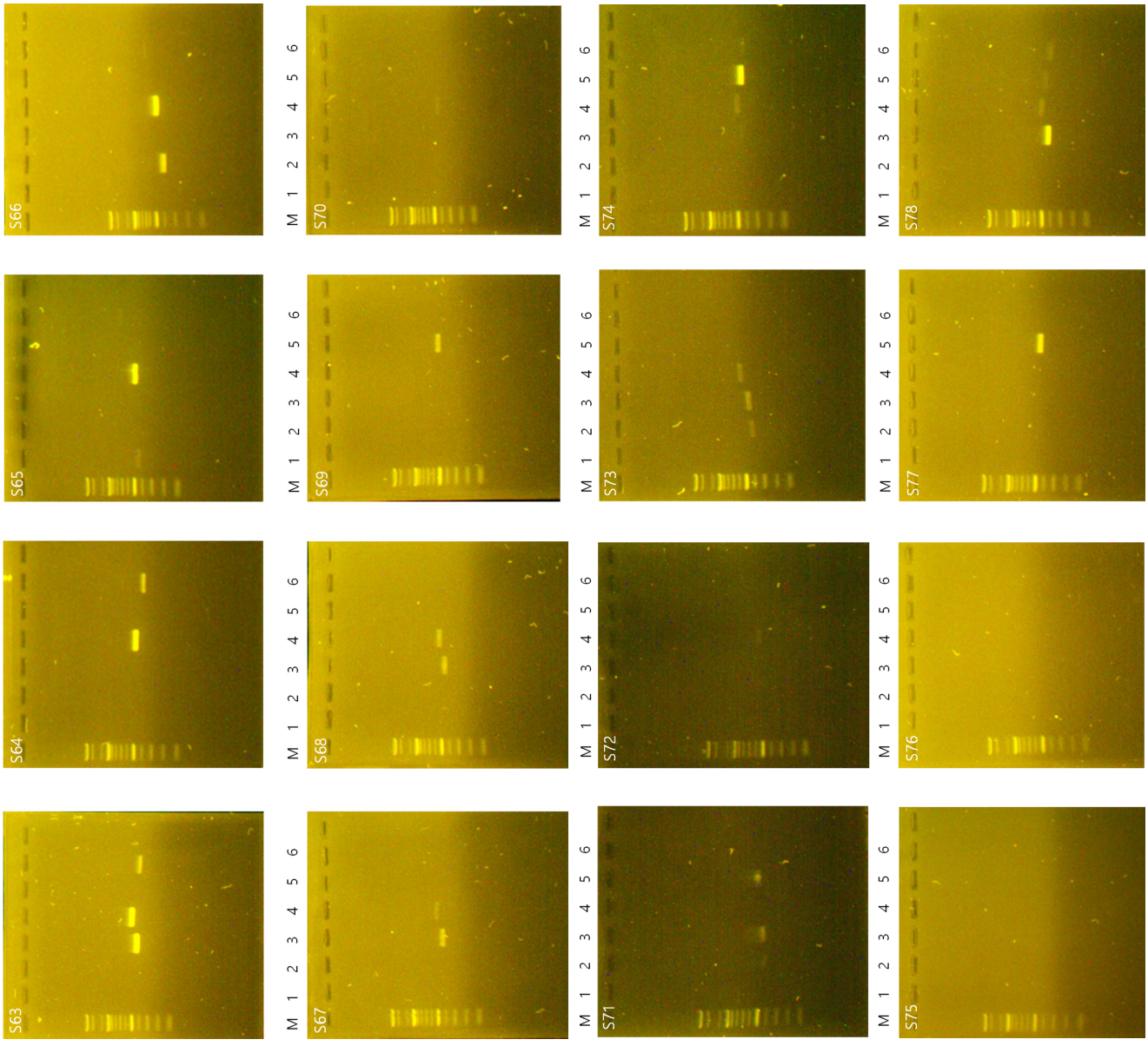
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S69
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S77
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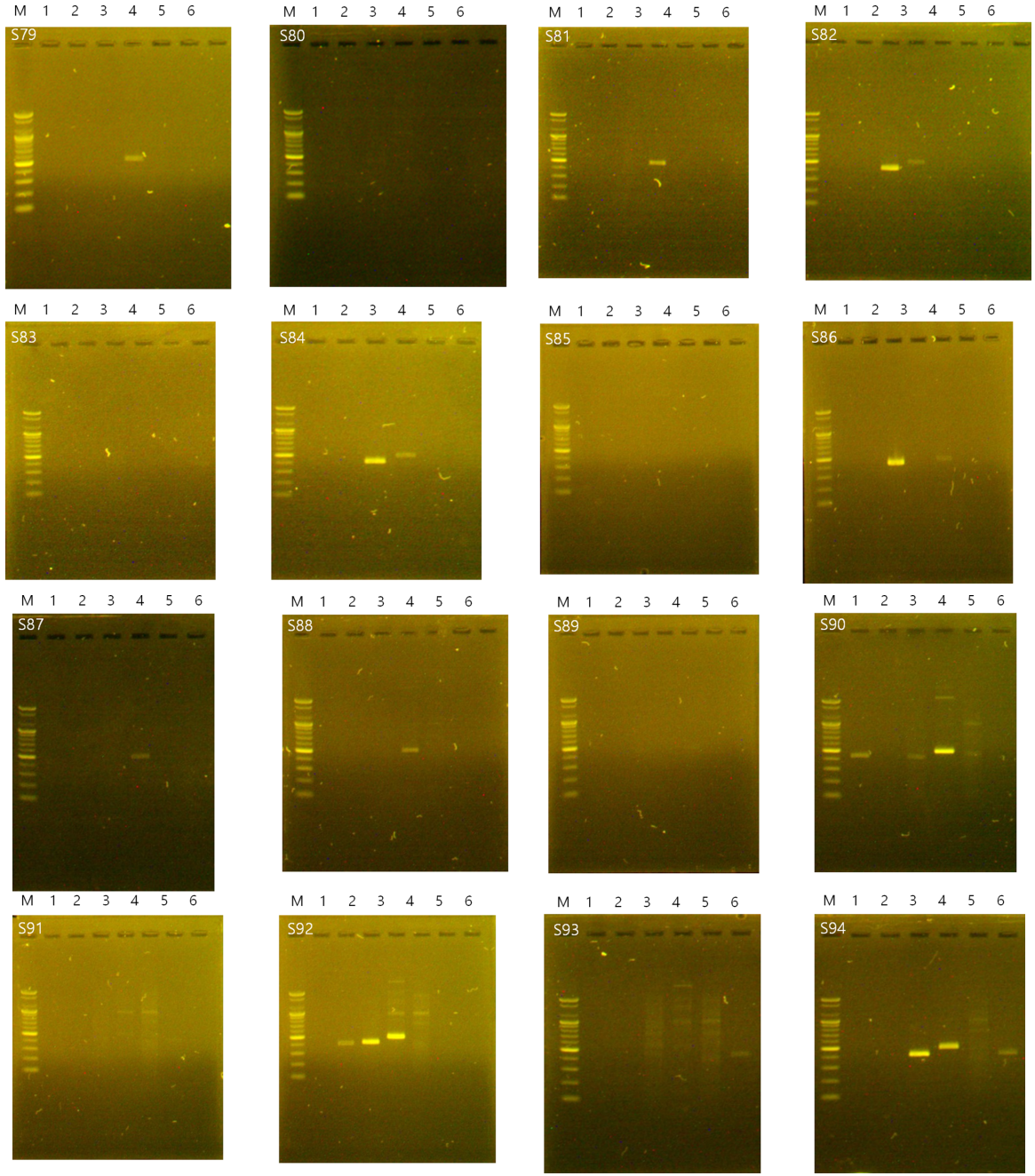
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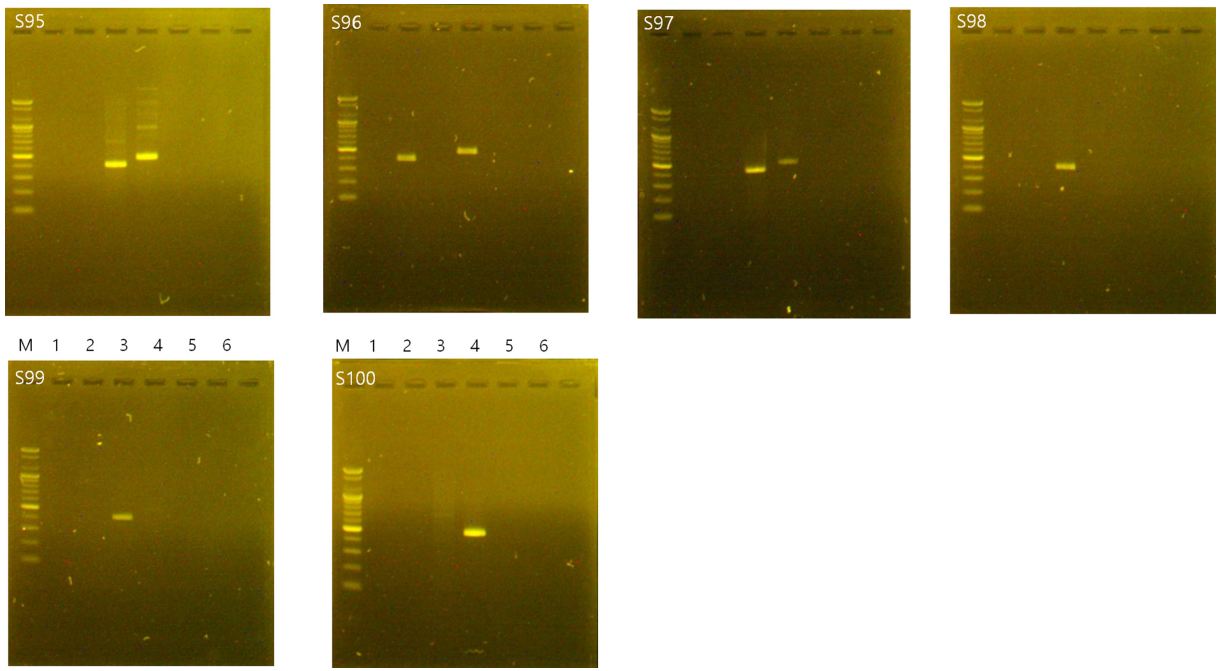

Supplementary Fig. 1. PCR amplification patterns of 100 commercial *Takifugu rubripes*. M: 100 bp DNA ladder; lanes 1-6: Ch1-5, Ch9-1, Ch18-9, Ch20-1, Ch20-4, and Ch20-13 deletions, respectively. S1-S100: Commercial samples listed in Table 1.