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Visual detection of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) simultaneously by duplex loop-mediated isothermal amplification

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ABSTRACT

Loop-mediated isothermal amplification (LAMP) is often confounded by the non-specific amplification arising from primer dimers, off-target priming, and other artifacts. Precipitation of the DNA produced during LAMP with the use of specific fluorescently labeled probe has proved the effectiveness in specific detection. Herein, two fluorophores (ROX and FAM) were attached to the primers S-LB-6 and R-FIP for Atlantic salmon and rainbow trout, respectively, which are self-quenched in unbound state and become de-quenched after binding to the dumbbell-shaped DNA specifically. The DNA precipitation and appearance of small sediment took 10 s of centrifugation at 1000 g, by adding polyethylenimine (PEI) 600. Each target species was specifically amplified with the predicted color of PEI-DNA sediment, namely red for Atlantic salmon, green for rainbow trout, and pale yellow for mixed species. The optimized duplex LAMP system has proved its specificity and can detect as little as 1 ng DNA in visual detection.

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) are the two most important salmonid species highly consumed worldwide. Given the limited availability of the wild resources, aquaculture production of these two species is increasing around the world, and the global production ranked 17th (0.814 million tons) and 9th (2.248 million tons) in 2016 for *O. mykiss* and *S. salar*, respectively, among all the major species produced in world aquaculture (FAO, 2020). In particular, *O. mykiss* is generally a freshwater-cultured fish species with the advantage of rapid growth and reproduction, while most *S. salar* is reared in seawater, with Norway, Chile, Scotland, and Canada the main producers among others.

Different preferences for the two salmonid species have been widely discussed and consumers are generally more inclined to *S. salar* which represents the excellent source of polyunsaturated fatty acids and essential micronutrients crucial for the balanced nutrition and optimal health (Sprague, Fawcett, Betancor, Struthers, & Tocher, 2020). Moreover, fresh *S. salar* with smooth taste and bright color is often served in raw by the means of *sashimi*. However, *O. mykiss* is generally not suitable for raw eating due to the health concerns by parasite infections and excessive accumulation of heavy metals (Barrientos et al., 2019; Xu et al., 2019). In addition, the freshwater-cultured *O. mykiss* was often reported to have earthy and musty off-flavors arising from the geosmin and 2-methylisoborneol (Lindholm-Lehto et al., 2019), and its price (about 20 RMB/kg, or 3 USD/kg) is generally much lower than *S. salar* (about 110 RMB/kg or 16 USD/kg) in China (author's note).

Traditionally, the identification of *O. mykiss* and *S. salar* can be achieved by the morphological traits, which, however, could become a challenging task when the product is quite complex or processed. To this end, driven by the high illegal profits, adulterating or substituting *O. mykiss* with much lower value into *S. salar* often occurs. For instance, Gu et al. (2020) highlighted *O. mykiss* from two smoked fish products (labeled as Atlantic salmon) collected from online retailers in China. Similar fraudulent conduct has also been identified in other countries, such as Spain (Muñoz-Colmenero, Juanes, Dopico, Martinez, & Garcia-Vazquez, 2017), and South Africa (Cawthorn, Steinman, & Witthuhn, 2012). To facilitate the regulation of such violation and the protection of

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Table 1
Primers information.

Species	Code	Primer sequence(5′-3′)	Reference
S. salar	S-F3	TCCGCCTCATATCAAGCCT	Li et al. (2022)
	S-B3	TGCCTCCAATTCAGGTAAGG	
	S-FIP	GGCGAGTACTCCGCCTAGTTTGTTTTACTTCCTATTCGCCTACGCA	
	S-BIP	CGTCCCCATCCTCCATACCTCTTTTTCGCTACCAGGGTCCAGAA	
	S-LB-6	GACTGACCTTTCGCCCACTC	
	S-LB-6-FAM	GACTGACCTTTCGCCCAC**T**C	
	S-BIP-1-ROX	CGTCCCCATCCTCCATACCTCTTTTTCGCTACCAGGGTCCAG**T**A	The present study
	S-LB-6-ROX	GACTGACCTTTCGCCCAC**T**C	
	S-FIP-B	Biotin-GGCGAGTACTCCGCCTAGTTTGTTTTACTTCCTATTCGCCTACGCA	
O. mykiss	R-F3	GTCCTCCCGTGAGGACAA	Xiong et al. (2020)
	R-B3	ACAGAAGGTGAAGGACCGTA	
	R-FIP	GTACTAGGGCGCCTCCTACGTATTTTCATTCTGAGGGGCCACTG	
	R-BIP	GGCTTCTCCGTTGACAACGCCTTTTCGGCTGCAATGACGAAGG	
	R-LB	ACTCTAACACGATTTTTCGCC	The present study
	R-FIP-FAM	GTACTAGGGCGCCTCCTACGTATTTTCATTCTGAGGGGCCAC**T**G	
	R-LB-FAM	ACTCTAACACGATTTTTCG**T**C	
	R-BIP-B	Biotin-GGCTTCTCCGTTGACAACGCCTTTTCGGCTGCAATGACGAAGG	

*__T__ denotes the site for FAM/ROX attachment.

consumers' interests, the development of robust and fast authentication methods to identify *O. mykiss* and *S. salar* is of paramount importance.

Till now, various methods have been applied for the detection of adulteration in fish products, and among them, nucleic acid-based assays are the most widely used due to their strong specificity and high sensitivity (Böhme, Calo-Mata, Barros-Velázquez, & Ortea, 2019). In particular, nucleic acid amplification tests (NAATs) have become an indispensable tool, where a few copies of DNA fragments can be exponentially amplified by the use of a thermal cycler since the revolutionary development of the polymerase chain reaction (PCR). Moreover, in the course of the last 10 years, a great variety of PCR-based analytical methods have been applied for fish authenticity studies (Chen et al., 2021; Kotsanopoulos et al., 2021; Shi et al., 2020). Nevertheless, these PCR-based methods generally require expensive equipments and are time-consuming to perform, precluding the facile implementation of these techniques for on-site or rapid testing.

Isothermal NAATs can avoid the need of thermal cycling and enable the DNA amplification at constant and moderate temperatures with great specificity and sensitivity, even being promising alternatives to PCR (Lee, 2017). In particular, as a rapid, simple, and cost-effective analytical method, loop-mediated isothermal amplification (LAMP) has been well-characterized and has demonstrated its prevalence among all isothermal NAATs (Becherer et al., 2020). Generally, LAMP uses four to six specially designed primers to recognize six to eight distinct target gene sequences, and with the help of specific DNA polymerase, the amplification can be performed under isothermal conditions (60–65 °C) in less than 60 min (Tsugunori et al., 2000). The accumulation of amplified DNA can be up to 10^9 copies, and correspondingly a large amount of white magnesium pyrophosphate precipitate enables facile visual detection even in a closed-tube format (Zhang, Lowe, & Gooding, 2014). Moreover, colorimetric indicators, such as calcein, hydroxynaphthol blue, SYBR Green and SYTO 9, can also be incorporated to visually detect the amplification (Scott, Layne, O Connell, Tanner, & Landers, 2020). Since the inception and development of LAMP in 2000, the technique has been established for a wide interdisciplinary applications, and specifically in the field of food safety assurance, LAMP has been applied for the detection of food-borne pathogens, allergens, fish/meat adulteration and mislabeling, and genetically modified organism (Huang et al., 2020). Recent studies have also exploited LAMP method for fish authenticity purpose, including the rapid identification of European eel (*Anguilla anguilla*) (Spielmann et al., 2019), *S. salar* (Xiong et al., 2021) and codfish (Li, Xie, Yu, Wang, & Chen, 2021).

In practice, LAMP also has deficiencies and a major disadvantage is the increased likelihood of false positives arising from non-specific amplification (Becherer et al., 2020). Compounding this problem is the fact that it is a challenging task for the traditional detection methods, listed above, to directly check the nonspecific amplification. The lack of target specificity further means that the above detection techniques cannot be multiplexed to allow detection of more than one target in a single reaction. To this end, target-specific detection of LAMP amplicons was widely discussed, mainly through the use of target-specific probes or modified primers as biorecognition elements (Varona & Anderson, 2019). Recent work investigating the use of a simple non-FRET (fluorescence resonance energy transfer) based approach with LAMP, whereby the fluorophores quenches/de-quenches in absence of a dedicated quencher (Gadkar, Goldfarb, Gantt, & Tilley, 2018), suggested that the problem of non-specific amplification could be solved for real-time assays through its use. Moreover, our previous work also succeeded the application of self-quenching fluorogenic approach for rapid identification of *S. salar* and the real time fluorescence curves can be observed only if the modified loop primer bound to the dumb bell shaped DNA specifically (Li et al., 2022). While the aforementioned self-quenching approach has been shown with real-time detection, it requires further study and modification for use in resource-limited settings. The ideal situation should be capable of detecting specific amplification sequences, and produce results that can be easily visualized.

Herein, taking advantage of the facile formation of an insoluble complex between DNA and cationic polyamines, for instance, polyethylenimine (PEI) (Mori, Hirano, & Notomi, 2006), a novel method (PEI-sqLAMP, self-quenching based LAMP) was established to achieve the visual and sequence-specific detection of *O. mykiss* and *S. salar*. With this assay, the fluorescence of self-quenching fluorogenic primers and the precipitation of the PEI-LAMP amplicon complex was combined, and the sediment exhibits a bright fluorescent emission only if the specific amplicons were obtained. Moreover, given the different fluorescence colors corresponding to the used fluorophores, a duplex visual system was also established to identify *O. mykiss* and *S. salar* simultaneously. To the best of our knowledge, this is the first work to achieve visual detection of specific LAMP products, under both simplex and duplex operations, enabling a facile application in point-of-care or resource-limited setting.

2. Materials and methods

2.1. Samples collection

Authentic specimens belonging to 18 fish species in our previous

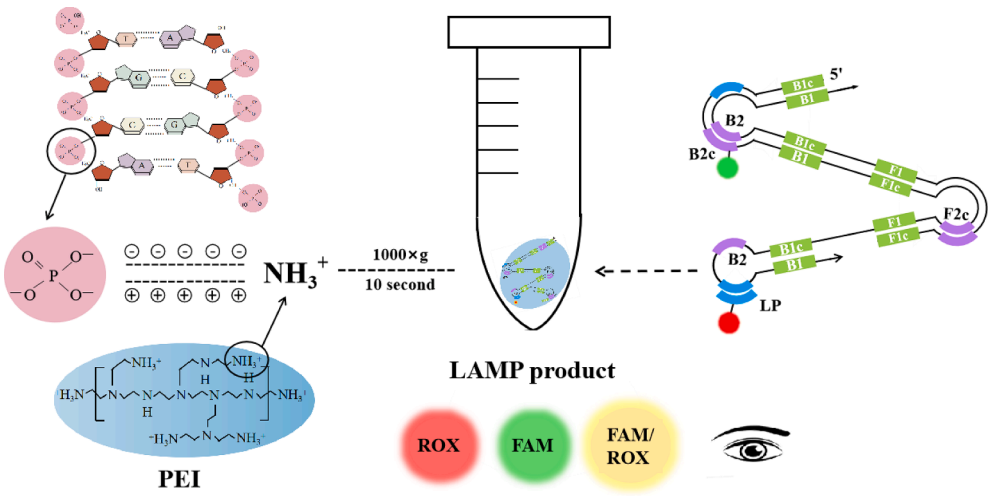

Fig. 1. Schematic illustration of the visual and sequence-specific detection of LAMP products. ROX and FAM represent the fluorescently labeled primers. At the end of amplification, for the positive sample, precipitated by certain amount of PEI 600, the ROX-labeled LAMP products appear red fluorescence, while the FAM-labeled LAMP products appear green fluorescence. For duplex LAMP assay, the existence of both ROX-labeled LAMP products and FAM-labeled LAMP products appear pale yellow fluorescence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

work (Xu et al., 2021) were also used in the present study, including *S. salar*, *O. mykiss*, *O. keta*, *O. masou*, *O. nerka*, *O. gorbuscha*, *O. tshawytscha*, *Gadus chalcogrammus*, *Melanogrammus aeglefinus*, *Lateolabrax japonicus*, *Epinephelus costae*, *Pennahia argentata*, *Upeneus japonicas*, *Lates niloticus*. Moreover, for method validation, 40 commercial fish products were randomly collected from the local markets in Nanjing, China. Label evaluation found the Chinese fish common name of *Da Xiyang San Wenyu* reported on the package for 20 products (namely s1-s20), with the rest 20 products generally reporting *Hong Zunyu* or *Guochan San Wenyu* (namely r1-r20) (Table S1). In spite of the absence of specific provisions to label fish products and the unavailability of a standardized fish nomenclature in China, all these names have been widely accepted on behalf of Salmonids (Xiong et al., 2016).

2.2. DNA extraction

Total DNA extraction was performed using the TIANamp Genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. DNA quality and concentration were determined using a BioPhotometer D30 (Eppendorf, Hamburg, Germany) spectrophotometer, reading all samples at 230 nm (A230), 260 nm (A260) and 280 nm (A280). For each sample, a standard working concentration of 50 ng/μL was prepared.

2.3. Designing of the fluorescently labeled primers

The LAMP primers (BIP, FIP, B3, F3, LB) specific for *S. salar* and *O. mykiss* were obtained from our previous works, with only one exception of R-LB designed in the present study (Table 1). The self-quenching element was designed by labeling an internal thymine (T) residue of the LAMP primer with 6-carboxyfluorescein (FAM) and carboxy-X-Rhodamine (ROX) (Gadkar et al., 2018). During the method validation, both ROX and FAM were attached on *S. salar* primers. For the development of the duplex LAMP system, *S. salar* and *O. mykiss* primers were labeled with ROX and FAM, respectively.

2.4. LAMP amplification

The simplex LAMP reaction was performed in a total reaction volume of 20 μL, including 0.8 μM of each inner primer, 0.4 μM loop primer, 0.2 μM of each outer primer (Table 1), 0.45 mM dNTP mix, 2.5 μL 10 × ThermoPol buffer, 2 mM $MgSO_4$, 8 units of *Bst* DNA polymerase (New England BioLabs, MA, USA) and 50 ng of DNA template. For the comparative study, 0.25 μM intercalating dye SYTO 9 was also added in the reaction mix. For the duplex LAMP system, the primer quantity was optimized (inner primers (0.8 μM), loop primer (0.4 μM), outer primers (0.2 μM) of both species, at ratios of 5:5, 4:6, 3:7, 2:8) and the primer sets with the optimal amplification efficiency for both species were used in the final mix.

The reaction was performed in a LightCycler® 96 real time PCR system (Roche, Switzerland) under the following conditions: 64 °C for 60 min (one cycle per min), followed by heating to 85 °C for 2 min (one cycle per min). Fluorescence signals were measured at the end of each cycle and a sigmoid shaped fluorescence curve could be observed for successful amplification. The real-time LAMP products were interpreted by amplification curve with respect to time (Ct value). One Ct is equal to one min.

Endpoint visual detection was carried out via the addition of a 0.2 μL SYBR Green I (10,000×) (Solaribo, Beijing, China), mixed thoroughly by shaking the tube. The positive LAMP result can be determined by direct observation of a color change from orange to green under ultraviolet (reaction tubes were laid directly on the UV transilluminator). Besides this, PEI was also used for endpoint visual detection, where the insoluble PEI-DNA complex exhibits bright fluorescent green and red colors with a transilluminator, for FAM-labeled and ROX-labeled, respectively.

Finally, for LFD (Lateral flow dipstick) detection, the primers S-FIP and R-BIP were labeled with biotin at the 5′-end (Table 1). After the amplification, 5 μL of the LAMP product was applied onto the commercial LFD strip (Beiji BioTech, Nanjing, China), standing for 5 min. For positive samples, two red lines should present on the LFD strip, while only the red control line was observed for the negative samples.

3. Results and discussion

3.1. Strategy for the visual and sequence-specific detection of the LAMP products

A sequence-specific and PEI-based loop-mediated isothermal amplification assay, namely PEI-sqLAMP, was developed to offer a rapid and visual detection of *O. mykiss* and *S. salar*, individually or simultaneously (Fig. 1). Particularly, LAMP primers (FIP, BIP, F3, B3 and LB) were obtained from our previous studies (Li et al., 2022; Xiong et al., 2020), with one exception of R-LB designed in the present work (Table 1). In order to meet the labeling criteria in self-quenched approach (Gadkar et al., 2018), the 3′ end of S-BIP, R-FIP, and R-LB was slightly modified, and two different fluorophores (FAM and ROX) were attached (Table 1), which are self-quenched in unbound state and become de-quenched after binding to the dumbbell-shaped target DNA specifically (Li et al., 2022). FAM and ROX are chosen because the appeared green and red fluorescence for the positive sample enables a facile visual distinguishing (Qian et al., 2018). Their feasibility was confirmed using *S. salar* as a case study before further investigation (Fig. S1).

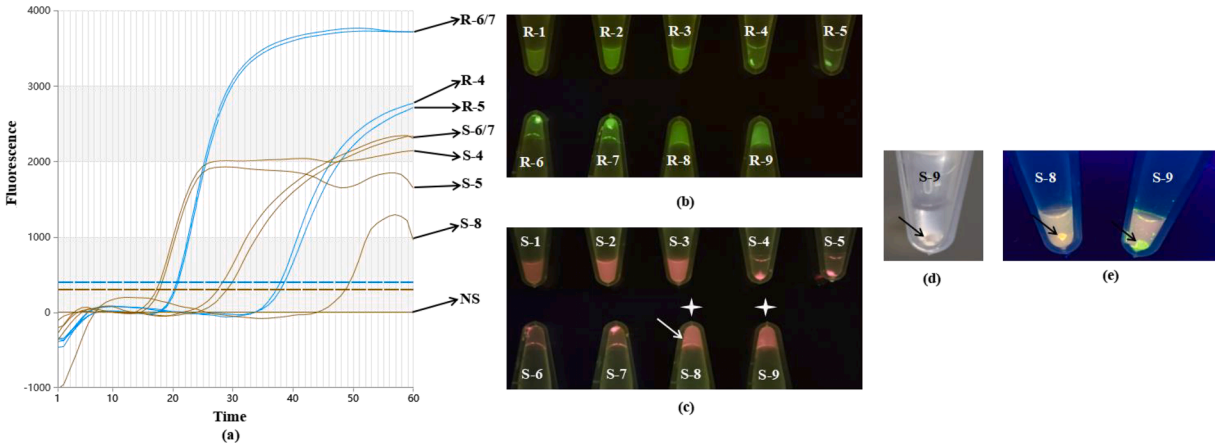

Fig. 2. Real time fluorescence curves **(a)** and visual detection **(b–e)** of LAMP amplifications targeting *S. salar* **(S)** and *O. mykiss* **(R)**. LAMP primer set, S-F3\S-B3\S-FIP\S-BIP\S-LB-6-ROX, was applied on *S. salar* **(S-4/5)** and distilled water **(S-1)**; LAMP primer set, S-F3\S-B3\S-FIP\S-BIP-1-ROX\S-LB-6, was applied on *S. salar* **(S-6/7)** and distilled water **(S-2)**; LAMP primer set, S-F3\S-B3\S-FIP\S-BIP-1-ROX, was applied on *S. salar* **(S-8/9)** and distilled water **(S-3)**; LAMP primer set, R-F3\R-B3\R-FIP\R-BIP\R-LB-FAM, was applied on *O.mykiss* **(R-4/5)** and distilled water **(R-1)**; LAMP primer set, R-F3\R-B3\R-BIP\R-FIP-FAM\R-LB, was applied on *O.mykiss* **(R-6/7)** and distilled water **(R-2)**; LAMP primer set, R-F3\R-B3\R-BIP\R-FIP-FAM, was applied on *O.mykiss* **(R-8/9)** and distilled water **(R-3)**. **NS** represents the samples failing to produce fluorescence signal, including S-1, S-2, S-3, S-9, R-1, R-2, R-3, R-8, R-9. **S-8** in **(c)** was highlighted with asterisk, since the sediment is visible by adding SYBR under UV **(e)**. However, by adding PEI, no sediment is visible under sunlight (data not shown), and only a tiny particle becomes visible under UV **(c)**. **S-9** in **(c)** was highlighted with asterisk, since the sediment is visible by adding PEI under sunlight **(d)**, or by adding SYBR under UV **(e)**, and become invisible by adding PEI under UV **(c)**.

For *S. salar*, the earlier amplification signals and generally the greater sediments were observed when using loop primer (S-4/5) for fluorescent labeling than inner primer (S-6/7) (Fig. 2a, c), consistent with our previous work (Li et al., 2022). A possible reason could be the rising steric hindrance for inner primers after attaching the chemical moiety (ROX) to the nucleotide backbone (Gadkar et al., 2018).

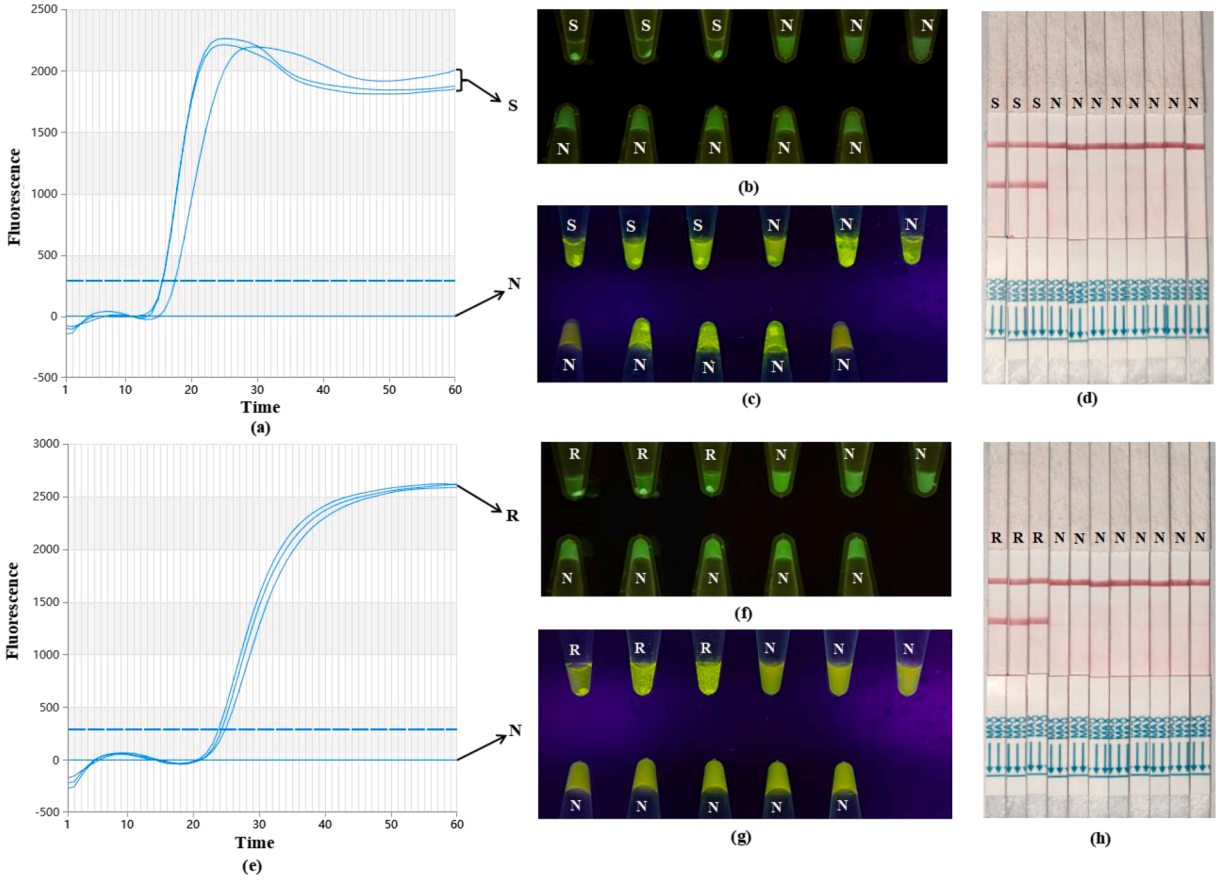

Fig. 3. LAMP amplification of *S. salar* **(S)**,*O. mykiss* **(R)**, and distilled water **(N)**. **(a)** and **(e)**: the real-time fluorescence curves for the amplification reaction; **(b)** and **(f)**: visual inspection by adding PEI; **(c)** and **(g)**: visual inspection by adding SYBR Green; **(d)** and **(h)**: visual detection on LFD strips. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

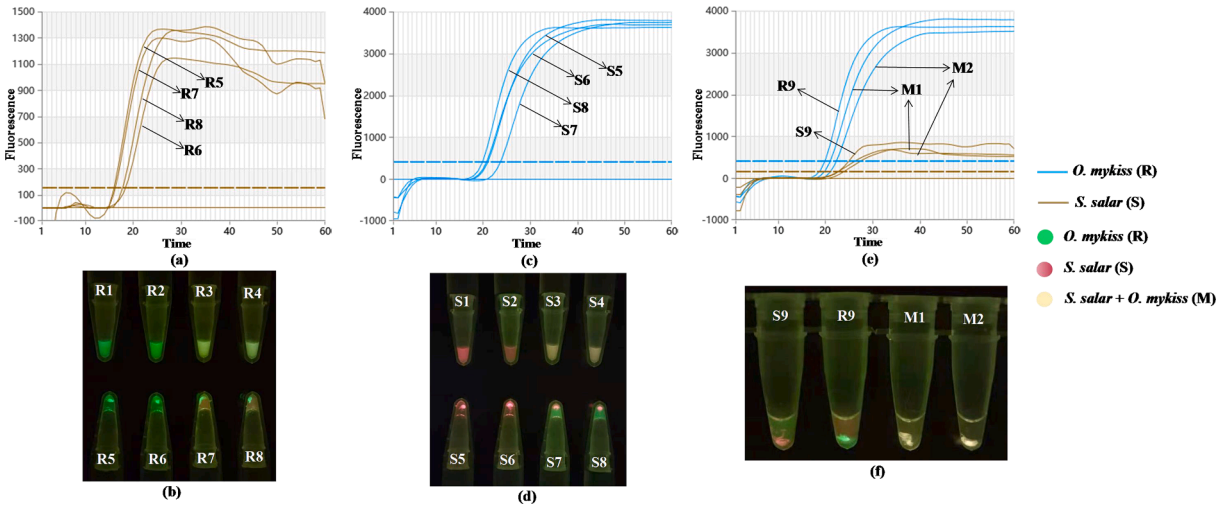

Fig. 4. Simplex and duplex LAMP amplification of *S. salar* **(S)**and *O. mykiss* **(R)**.The LAMP amplification of *O. mykiss* **(R5, R6)** and distilled water **(R1, R2)** in simplex system, monitored in real time fluorescence **(a)** and naked eyes **(b)**.The LAMP amplification of *O. mykiss* **(R7, R8)** and distilled water **(R3, R4)** in duplex system, monitored in real time fluorescence **(a)** and naked eyes **(b)**.The LAMP amplification of *S. salar* **(S5, S6)** and distilled water **(S1, S2)** in simplex system, monitored in real time fluorescence **(c)** and naked eyes **(d)**.The LAMP amplification of *S. salar* **(S7, S8)** and distilled water **(S3, S4)** in duplex system, monitored in real time fluorescence **(c)** and naked eyes **(d)**.The LAMP amplification of *S. salar* **(S9)**, *O. mykiss* **(R9)**, *S. salar* and *O. mykiss* **(M1, M2)** in duplex system, monitored in real time fluorescence **(e)** and naked eyes **(f)**.

However, the inner primers are generally used at twice the concentration of the loop primers in the LAMP reaction and thus incorporated to a higher degree into amplicons, which should theoretically produce a better signal than loop primer, as confirmed on RNA virus by (Ball et al., 2016) and also our work on *O. mykiss* (Fig. 2a, b). Despite the absence of harmonization on labeling position of the fluorophore, the enhancing effect for loop primers can be observed for both *S. salar* and *O. mykiss* (Fig. 2), consistent with the result of (Nagamine, Hase, & Notomi, 2002). In particular, without the loop primer, a delayed amplification and poor signal was highlighted for S-8 (Fig. 2a), and the sediment was only visible by adding both PEI and SYBR Green (Fig. 2c, e). The improved visibility by using SYBR Green could be attributed to the non-specific amplification, since SYBR Green can fluoresce strongly in the presence of any double-stranded DNA (Ye, Fang, Li, & Kong, 2018). The failure to obtain positive amplification curve (Fig. 2a) and PEI-complex sediment (Fig. 2b, c) for S-9, R-8, and R-9 highlighted also the necessity of loop primer, while the non-specific amplification makes the sediment visible in sunlight (Fig. 2d) and after adding SYBR Green (Fig. 2e) for S-9.

Precipitation of the DNA produced during LAMP can be achieved by using ethanol, isopropanol, polyethylene glycol (PEG) and PEI (Fig. S2). Since PEI with simple structure has strong electrostatic interaction with DNA by its large charge density (Osland & Kleppe, 1977), the sediments in the case of PEI were generally greater than other substances and PEI with the molecular weight of 600 was finally selected in the present study (Fig. S2a). While the non-specific amplicons could also be precipitated using PEI with the molecular weight of 1800 and 10000, as highlighted in negative controls (Fig. S2a). Moreover, to facilitate the formation of PEI-DNA complex precipitate, the optimal parameter was to add PEI 600 of 3 μL (Fig. S2b), followed by centrifugation at 1000 g for 10 s (Fig. S3). The insoluble PEI-DNA complex is visible by naked eye, and exhibits bright fluorescent green and red colors with a trans-illuminator, for FAM-labeled and ROX-labeled, respectively (Mori et al., 2006). To this end, the individual species can be detected by simply reading the green or red color, while the existence of mixed species makes the sediment a pale yellow color (Fig. 1). Finally, the sediments were also visible for the LAMP amplifications using DNA template of poor quality (A260/A280 and A260/A230 less than 1.8) (Fig. S4), consistent with the opinion about the high tolerance to impurities (Spielmann et al., 2019).

In order to evaluate the specificity of the visual detection method, twenty-two LAMP reactions, including six positive controls (*S. salar* and *O. mykiss*), and sixteen negative controls (distilled water) were performed. According to the assays using sequence-independent SYBR Green (Fig. 3c, g), six false positive results were obtained from the *S. salar* system using distilled water, indicating the occurrence of non-specific amplification, which has also been revealed in previous study (Ye et al., 2018). With the aim to differentiate specific amplification from the undesired amplification, two common strategies were available: 1) target-specific detection through the use of target-specific probes or modified primers as biorecognition elements (Tsugunori et al., 2000), 2) avoid to trigger the primer aggregation by adding gold nanoparticle, graphene oxide, and self-avoiding primers (Özay & McCalla, 2021). Generally, the former one is more reliable and cost-effective, and even opens up new application opportunities in the field of multiplex detection. Among them, self-quenching approach is a promising technique and has proved itself a useful tool to avoid the detection of non-specific amplification (Gadkar et al., 2018; Li et al., 2022). As shown in Fig. 3(a, e, d, h), typical amplification curve and the crimson red bands at both control and test lines can only be obtained from positive controls, highlighting the specificity, which would not be compromised by the visual detection of the PEI-LAMP amplicon sediment (Fig. 3b, f).

3.2. Duplex PEI-sqLAMP assay for visual and sequence-specific detection

Generally, due to the complicated structure with LAMP products and the large quantities of LAMP primers, multiplex LAMP is a quite challenging task (Mayboroda, Katakis, & O'Sullivan, 2018). Herein, taking advantage of the difference with the fluorophore color (red for ROX, green for FAM), the feasibility of a duplex PEI-sqLAMP assay for simultaneous detection of *O. mykiss* and *S. salar* was validated (using the optimized temperature 64 °C, Fig. S5). In particular, with the aim to avoid the interference caused by the interaction of multiple primers, the optimization of the primer quantity in the final reaction was performed by adding primers together at several quantities. Real time fluorescence and visual detection highlighted an acceptable efficiency for *S. salar* and *O. mykiss* by adding 0.1 μM of S-F3 and S-B3, 0.4 μM of S-FIP and S-BIP, 0.3 μM of S-LB-6-ROX, 0.2 μM of R-F3 and R-B3, 0.8 μM of R-FIP-FAM and R-BIP, 0.4 μM of R-LB (Fig. 4e, f). Moreover, there was no significant difference between simplex and duplex LAMP for both species in the real

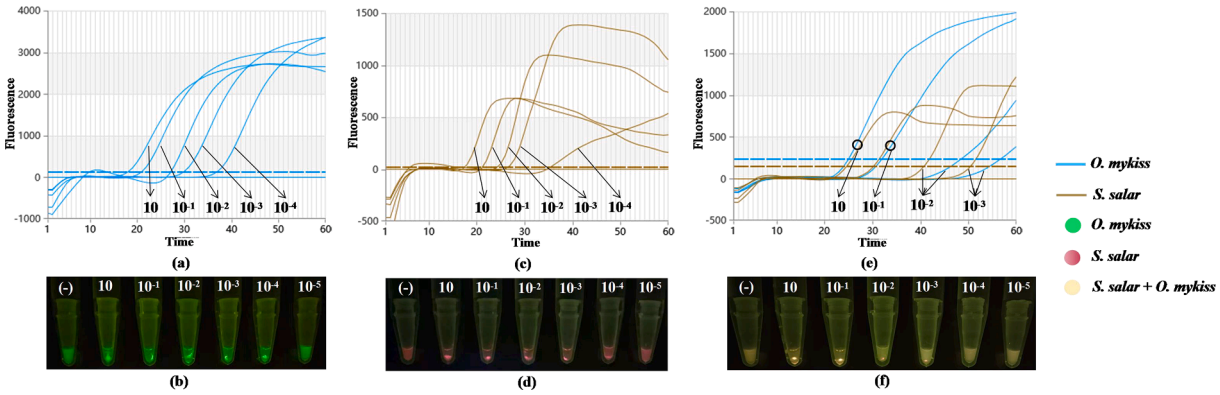

Fig. 5. Sensitivity evaluation of the simplex **(a, b, c, d)** and duplex **(e, f)** LAMP assay, observed using real time fluorescence **(a, c, e)**, and naked eyes **(b, d, f)**.The 10-fold serial dilutions of *O. mykiss* **(a, b)** and *S. salar* **(c, d)** DNA (from 10 ng to 0.1 pg) were prepared. For some cases, no amplification curve was observed with the dilutions of 10^{-4} and/or 10^{-5}, and the negative control, which thus were not marked in the figures. The minimum amount of the detectable DNA for duplex LAMP should be 1 ng in visual detection, despite the weak red sediment with 10^{-2} and 10^{-3} **(f)**. While the amplification curves highlighted the minimum amount of the detectable DNA for duplex LAMP should be 0.01 ng in real time fluorescence **(e)**. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

time fluorescence curve (Fig. 4a, c) and PEI sediment (Fig. 4b, d). Despite the low fluorescence signal for *S. salar* in the duplex system (Fig. 4e), an increased volume with *S. salar* primers would lead to the amplification failure with *O. mykiss* (data not shown). The results of visual detection in duplex LAMP were consistent with simplex one, where PEI-DNA complex become red for *S. salar*, green for *O. mykiss*, and pale yellow for mixed species (Fig. 4f).

The sensitivity was characterized by the lowest amount of DNA that can yield a detectable signal. To this end, 10-fold serial dilutions of genomic DNA from 10 ng to 0.1 pg were performed (Fig. 5) and the minimum amount of the detectable DNA reached 1 pg for simplex LAMP, which increased up to 0.01 ng and 1 ng for duplex LAMP using the realtime fluorescence and visual detection, respectively. The improved sensitivity with PEI-sqLAMP, compared with sequence-independent SYBR Green (Xiong et al., 2021), was consistent with previous study (Li et al., 2022). Moreover, a comparative evaluation of the absolute limit of detection using different detection methods also highlighted the poorer sensitivity with sequence-independent methods (Table S2), and a possible reason could be the reduced efficiency by non-specific amplification (Wang, Brewster, Paul, & Tomasula, 2015). While the inferior sensitivity with the duplex system confirmed the difficulty to build multiplex LAMP.

The specificity of the duplex PEI-sqLAMP assay was verified using the genomic DNA of 6 closely-related salmonid species and other 12 commercially important fish species commonly encountered in food-stuffs (belonging to Gadiformes, Perciformes, Pleuronectiformes, and Cypriniformes). Each target species was specifically amplified with the predicted color of PEI-DNA sediment (Fig. S6). While all the other 18 non-target species failed the amplification and no PEI-DNA sediment is visible. The application of the duplex PEI-sqLAMP assay on 40 commercial products highlighted *S. salar* for 19 products (red sediment), *O. mykiss* for 12 products (green sediment) (Fig. S6). The results were cross-confirmed by DNA barcoding method (Table S1), where *S. salar* and *O. mykiss* were only identified from the samples positive in the LAMP assay. The rest 9 samples negative in the LAMP assay were identified as *O. gorbuscha* and *O. keta* (Table S1).

4. Conclusions

With the results of the present study, a LAMP assay using self-quenching approach and PEI for visual and specific detection of *S. salar* and *O. mykiss*, individually and simultaneously, was developed. Each target species was specifically amplified with the predicted color of PEI-DNA sediment, namely red for *S. salar*, green for *O. mykiss*, and pale yellow for a mix species. With the novel assay, as little as 1 pg of *S. salar* and *O. mykiss* DNA could be specifically detected in simplex system, which increased up to 0.01 ng and 1 ng in duplex system. Finally, the duplex PEI-sqLAMP assay was successfully applied on commercial products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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