A quantitative PCR based environmental DNA assay for detecting Atlantic salmon (*Salmo salar* L.)

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Abstract

1. The Atlantic salmon (*Salmo salar* L.) has worldwide ecological, cultural and economic importance. The species has undergone extensive decline across its native range, yet concerns have been raised about its invasive potential in the Pacific. Knowledge on the distribution of this species is vital for addressing conservation goals.
2. This study presents an eDNA assay to detect *S. salar* in water samples, using quantitative PCR (qPCR) technology. Species-specific primers and a minor groove binding (MGB) probe were designed for the assay, based on the mitochondrial cytochrome oxidase I (COI) gene.
3. The results of this study indicate that eDNA is a highly effective tool for detecting *S. salar in situ*, and could provide an alternative, non-invasive method for determining the distribution of this species.

Keywords: distribution, fish, monitoring, new techniques, river.

1. Introduction

The Atlantic salmon, (*Salmo salar* L.), is of ecological, cultural and economic importance. As a result, this species has been the subject of intense exploitation ranging from commercial fisheries, recreational fishing and intensive aquaculture (Morton, Ariza, Halliday, & Pita, 2016; Piccolo & Orlikowska, 2012). Although *S. salar* is protected under Annex II and Annex V of the EU Habitats Directive, and efforts to reduce fishing pressure and restore freshwater habitats

have been implemented, this once abundant species has continued to decline (Chaput, 2012; Friedland et al., 2009). Numerous factors including recruitment failure at sea (Chaput, 2012; Friedland et al., 2009), obstacles to migration in freshwater (Thorstad, Økland, Aarestrup, & Heggberget, 2008) and pollution from agricultural, industrial and urban sources (Hendry, Cragg-Hine, O'Grady, Sambrook, & Stephen, 2003) have contributed to the deterioration of *S. salar* populations. Furthermore, the species is used for intensive aquaculture outside its native range. Large escapes of *S. salar* happen with regularity in these areas, causing concerns about the species' invasive potential (Fisher, Volpe, & Fisher, 2014; Piccolo & Orlikowska, 2012). To adequately address these issues, and to achieve the conservation objectives of the species, it is vital to have knowledge on its distribution. At present, *S. salar* monitoring involves electrofishing surveys, the placement of fish counters or traps, rod catch data provided by anglers and redd counts (The Standing Scienctific Commitee on Salmon, 2016). These surveys can be expensive, labour intensive and also potentially harmful to the fish (Snyder, 2004). Clearly, there is a need for an effective, efficient and non-invasive sampling method to monitor the species. To this end, environmental DNA (eDNA) analysis may provide an alternative sampling strategy for monitoring the distribution of *S. salar* for management and conservation purposes. Environmental DNA is the collective term for DNA present freely in the environment which has been shed by organisms (in the form of mucus, faeces, gametes or blood, for example), and can be extracted (Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012; Thomsen & Willerslev, 2015). It has been shown to be an effective method for detecting species in freshwater (Carlsson et al., 2017; Clusa, Ardura, Fernández, Roca, & García-Vázquez, 2017; Gustavson et al., 2015), marine (Gargan et al., 2017) and terrestrial (Willerslev, 2003) environments. Furthermore, eDNA has been shown to be a useful tool for detecting rare species in freshwater habitats. For example, Boothroyd, Mandrak, Fox and Wilson (2016) successfully detected the threatened spotted gar (*Lepisosteus oculatushas* Winchell) in sites where the species was thought to be extirpated.

Recent studies have developed and deployed specific primers for the detection of *S. salar* in eDNA water samples. A study by Clusa et al. (2017), for example, developed *S. salar*–specific primers using the 16S ribosomal DNA (rDNA) region. These authors successfully identified *S. salar* in their eDNA samples using PCR-RFLP (Polymerase chain reaction- restriction fragment length polymorphism). Alternatively, Dalvin, Glover, Sørvik, Seliussen and Taggart (2010) utilised the mitochondrial DNA (mtDNA) cytochrome c oxidase (COI) gene for their primer development, followed by traditional PCR analysis. While the COI primers in this study

were successful in amplifying DNA from tissue samples (both fresh and degraded) the authors were unable to detect *S. salar* DNA in their eDNA samples (Dalvin et al., 2010). The assay presented here provides an improvement on these studies. As well as developing species-specific primers with the COI gene, the present assay incorporates an additional species-specific minor groove binding (MGB) probe which allows the eDNA sample to be analysed in quantitative PCR (qPCR). Furthermore, the MGB probe allows for additional sensitivity and specificity of the assay, as three sequences as opposed to two are checked against the target template DNA (Herder et al., 2014).

The aim of this study was to develop an MGB based qPCR assay to detect the presence of *S. salar*. As observed in other studies (Laramie, Pilliod, & Goldberg, 2015) this approach may also allow for the detection of *S. salar* populations in locations where they have not been recorded with traditional methods.

2. Methods

2.1 eDNA qPCR assay development

Primer Express 3.0 (Applied Biosystems-Roche, Branchburg, NJ) was used to design the species-specific primers (forward primer: 5'-CGC CCT AAG TCT CTT GAT TCG A-3', and reverse primer 5'-CGT TAT AAA TTT GGT CAT CTC CCA GA-3') and 5' NED labelled TaqMan® minor groove binding probe (5'-AGA ACT CAG CCA GCC TG-3') for *S. salar*, which targeted the mtDNA COI region. The total amplicon size, including primers, was 74 base pairs. Probe and primer sequences were matched against the National Centre for Biotechnology Information (NCBI - http://www.ncbi.nlm.nih.gov/) nucleotide database with BLASTn (Basic Local Alignment Search Tool) to verify the species specificity for the *in silico S. salar* assay. The *S. salar* assay was tested *in vitro* with both closely related and other fish species (marine and freshwater) including brown trout (*S. trutta*), sea lamprey (*Petromyzon marinus* L.), pink salmon (*Oncorhynchus gorbuscha* Walbaum) and herring (*Clupea harengus* L.) to ensure the assay did not amplify other fish species. The qPCR assay was optimized using tissue extracted from *S. salar*.

2.2. Study area and field validation of *S. salar*

Three salmonid rivers located in the south of Ireland were selected for field validation of the eDNA assay: the Dinin, Burren and Dalligan rivers (Table 1, Figure 1). Each of these rivers contains an obstacle or barrier, which has the potential to prevent or delay the migration of *S. salar*. The Dinin and Burren rivers are tributaries of the Nore and Barrow rivers respectively,

which are located in the south east of Ireland. The Nore and Barrow rivers are classified as Special Areas of Conservation (SAC) under the EU Habitats Directive, with *S. salar* qualifying as a species of interest in both catchments. Conversely, the Dalligan river is a relatively smaller system without SAC status. It does, however, have the potential to hold *S. salar* populations, at least below the obstacle that was assessed in this study because it is the lower most obstacle in the river (approximately 2km from the sea). The obstacles on the Dalligan and Dinin rivers did not have fish passes, however a salmonid fish pass was present on the obstacle in the Burren river. Electrofishing was carried out by Inland Fisheries Ireland upstream and downstream of each obstacle in July 2017 to verify the presence or absence of *S. salar* at each site. Environmental DNA samples were collected on the same day that the electrofishing was carried out, prior to any individuals entering the river.

2.3. eDNA collection, filtering and extraction

Environmental DNA samples were collected from each river in sterilized 2L containers, and filtered in the field using a peristaltic pump. Three replicate eDNA samples were collected both upstream and downstream of each river obstacle. One negative field control per location (upstream and downstream) consisting of ddH_20 was also filtered, resulting in a total number of six eDNA samples and two field controls collected per river. Environmental DNA was collected on 47 mm glass microfiber filters (1.5 µm) and placed into 2.0 mL Eppendorf tubes prior to being frozen at -20˚ C. All work with eDNA was carried out in a dedicated Low Copy DNA laboratory to reduce contamination risk. Environmental DNA was extracted using a modified version of the CTAB (cetyltrimethylammonium bromide) protocol (Möller, Bahnweg, Sandermann, & Geiger, 1992). One-half of a glass microfiber filter was placed into a new 2.0 mL Eppendorf tube, to which 750 µL of CTAB buffer (100 mM Tris-HCL, 20 mM EDTA, 1.4 M NaCl, 2% CTAB), and 7 µL of Proteinase K (20 mg mL^{-1}) was added. Samples were vortexed for 10 seconds and incubated at 56˚ C for 2 hours, after which 750 µL of Phenol/Chloroform/Isoamyl Alcohol (25:25:1 v/v) was added. Samples were manually mixed for 15 seconds and centrifuged (11,000 x g, 20 min). The aqueous phase was transferred to a new tube containing 750 µL of Chloroform/Isoamyl Alcohol (24:1 v/v), the manual mixing and centrifugation steps were repeated, and the aqueous phase was transferred to a new tube. The eDNA was then precipitated by adding one volume of isopropanol alcohol to the aqueous phase and incubating the mixture at -20˚ C for 1 hour, and then centrifuged (11,000 x g, 20 min). The pellets were washed with 750 µL of 70% ethanol and centrifuged (11,000 x g, 5

min). The ethanol was carefully removed, and the pellets dried in a heating block (50˚ C, 5 min) before resuspending the eDNA in molecular-grade water.

2.4. eDNA assay deployment

Environmental DNA concentrations were determined by qPCR using an Applied Biosystems ViiA™ 7 (Life Technologies, Inc., Applied Biosystems, Foster City, CA) quantitative thermocycler. The qPCR reaction was conducted in a final reaction volume of 30µL, comprised of 15 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems, Foster City, CA), 3 µL of each primer (final concentration of 2 µM), probe (final concentration of 2 µM), DNA template (3 µL) and ddH_2O. Warm-up conditions of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles between 95°C for 15 s and 60°C for 1 min were used for the qPCR run. DNA extracted from *S. salar* tissue (quantified with NanoDrop®-1000, Thermo Scientific, Wilmington, DE) was used to generate the standard curve using seven 10:1 serial dilutions. Concentrations for the serial dilution ranged from 3ng/µL to 3 x 10^{-6} ng/µL. The eDNA field samples were run on two separate 96-well clear qPCR plates. Each plate had 3 no-template controls (NTCs) to ensure no contamination occurred during the preparation of the qPCR plate. Individual standard curves were generated for each qPCR plate ($y = -3.32x + 19.968$, efficiency = 100.018%, $R^2 = 0.999$ (1) and $y = -3.25x + 20.091$, efficiency = 103.101%, $R^2 = 0.997$ (2)). All standard curve samples, field samples and controls were quantified in triplicate (three technical replicates). A positive detection was defined as being within the range of the standard curve, and when at least 2 out of the 3 technical replicates contained amplifiable DNA with Cq differences not exceeding 0.5. If the difference between 1 out of 3 technical replicates exceeded 0.5Cq, this technical replicate was excluded from the study. However, if the Cq value of 2 out of 3 technical replicates differed by more than 0.5Cq, that particular dilution series or field replicate was excluded from further study. As *S. trutta* was present in all rivers, both upstream and downstream of the obstacles (Table 1), this species was used as a positive field control to test for the presence of amplifiable DNA in sites where no *S. salar* was recorded during electrofishing surveys. The *S. trutta* assay from previously published work (Gustavson et al., 2015) was used on eDNA samples from above the bridge apron in the Dinin river, and above and below the weir in the Dalligan river. Three replicates per location with one technical replicate were used for this analysis.

3. Results and Discussion

The present assay was successful in detecting *S. salar* DNA *in silico*, *in vitro* and *in situ*. Zero amplification of closely related species (*S. trutta*) or any other species occurred with the *S. salar* MGB qPCR assay. The dynamic range of the standard curves was between 18.3 Cq and 37.4 Cq. The lowest detected eDNA concentration within the range of the standard curve was 0.016 ng L^{-1} at Cq 34.5 (average over 3 technical replicates, standard deviation 0.0015 ng L^{-1}). For the purposes of analysis, one technical replicate from the 1:7 serial dilution was disregarded (equation 1), and the entire 1:7 dilution for the standard curve (equation 2) was disregarded because differences in Cq values between either one or more technical replicates in these samples exceeded 0.5. For the remainder of the samples, however, the standard deviation between technical replicate Cq values ranged from 0.011 to 0.303.

The results of the eDNA analysis mirrored what was observed in the electrofishing surveys. At each site where the presence of *S. salar* was confirmed by electrofishing, its presence was confirmed by eDNA analysis (Table 2, Figure 2). At sites where *S. salar* was not detected by electrofishing, a negative result was also obtained in the eDNA samples when assessed with the *S. salar* assay (Table 2, Figure 2). However, detectable eDNA was confirmed at all sites including the sites where no *S. salar* DNA was detected, as amplification occurred when the same samples were run in qPCR with the *S. trutta* assay. No DNA was amplified in any of the NTCs or negative field controls.

The results of both the eDNA analysis and electrofishing surveys suggest that the bridge apron on the Dinin river is an impassable barrier for *S. salar*, and that *S. salar* is not present in the Dalligan river, at least in the sites surveyed. It is worth noting, however, that there is a possibility that *S. salar* could have been present, but in too low abundance/biomass to be detected with the assay presented here. This is unlikely, however, as the *S. salar* eDNA concentrations detected in this study, in particular downstream of the bridge apron in the Dinin river, were within the range of the standard curve. However, the phrase "low probability of occurrence" may be more appropriate than "absent" or "not present" (Baldigo, Sporn, George & Ball, 2016).

The assay reflected the electrofishing survey results, demonstrating the potential future use of this assay for detecting the species without traditional sampling methods. It is important to note, however, that at present it is not possible to derive details about the population structure, such as length frequency distributions and age structure (which is readily available with traditional sampling methods) with eDNA analysis (Evans, Shirey, Wieringa, Mahon &

Lamberti, 2017). While attempts have been made to model relationships between the density of species with eDNA concentration (Baldigo et al., 2016), this is inherently difficult in a river system, because eDNA may accumulate from numerous different sources upstream, and would require extensive sampling regimes.

While this study clearly demonstrates the value of eDNA as a tool for monitoring the impact of river obstacles on *S. salar,* it could be applied in numerous different contexts including monitoring *S. salar* escapes from fish farms outside the native range. Furthermore, this eDNA assay would be particularly valuable for monitoring *S. salar* year-round. Traditional sampling methods are typically carried out during specific times of the year. For example, redd counts are only possible during the spawning period, and electrofishing surveys are typically restricted to the summer months when water levels are low, and fish are not migrating. While fish counters and traps can provide year-round records of *S. salar* movements, the structures themselves can act as obstacles to the movement of other, non-salmonid fish. For example, resistivity fish counters are typically placed on sloping weir-like structures (Lucas & Baras, 2000) which have been shown to impede the movement of river lamprey *Lampetra fluviatilis* L. (Lucas, Bubb, Jang, Ha, & Masters, 2009; Russon, Kemp, & Lucas, 2011) and barbel *Barbus barbus* L. (Lucas & Frear, 1997). In addition, eDNA is potentially a more cost-effective and rapid approach to monitoring species, particularly when compared with multiple-pass electrofishing (Evans et al., 2017) and when sampling across large geographic areas is required (McKelvey et al., 2016).

To conclude, the assay presented here is an effective method of detecting *S. salar* in rivers. Similar to Laramie et al. (2015) the assay presented here could be used to identify new conservation areas for the species, and additionally, can provide evidence to support remediation action, for example removing river obstacles that may be preventing the migration of the species.

Acknowledgements

This research was funded by the Atlantic Salmon Trust (Salmo Slime project) with additional support from the Irish Environmental Protection Agency (Reconnect project). The authors would like to thank Inland Fisheries Ireland for carrying out all the electrofishing surveys which supported this research.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1. The different combinations of *S. salar* and *S. trutta* presence/absence downstream and upstream of the river obstacles listed. The occurrence of each species was confirmed by electrofishing.

River	**Obstacle Type**	***S. salar* Downstream**	***S. salar* Upstream**	***S. trutta* Downstream**	***S. trutta* Upstream**
Burren	Weir	Yes	Yes	Yes	Yes
Dalligan	Weir	No	No	Yes	Yes
Dinin	Bridge Apron	Yes	No	Yes	Yes

Table 2. The Cq values and eDNA concentrations (ng L^{-1}) (average over three technical replicates per site replicate) from the *S. salar* assay in each river. Average concentrations (± SD) are given for each location (upstream or downstream of the river obstacle).

River	Location	*S. salar* present	Site Replicate	Average Cq (n= 3 technical replicates)	DNA conc (ng L^{-1})
Burren	Downstream	Yes	1	34.064	0.023
			2	33.464	0.035
			3	33.861	0.026
				33.796 ± 0.31	0.028 ± 0.006
	Upstream	Yes	1	34.468	0.017
			2	34.553	0.016
			3	34.549	0.016
				34.523 ± 0.05	0.017 ± 0.001
Dinin	Downstream	Yes	1	32.616	0.043
			2	32.861	0.035
			3	33.569	0.021
				33.015± 0.5	0.033 ± 0.011
	Upstream	No	1	undetermined	undetermined
			2	undetermined	undetermined
			3	undetermined	undetermined
Dalligan	Downstream	No	1	undetermined	undetermined
			2	undetermined	undetermined
			3	undetermined	undetermined
	Upstream	No	1	undetermined	undetermined
			2	undetermined	undetermined
			3	undetermined	undetermined

Figure 1. Map showing the locations of the sampling sites in this study.

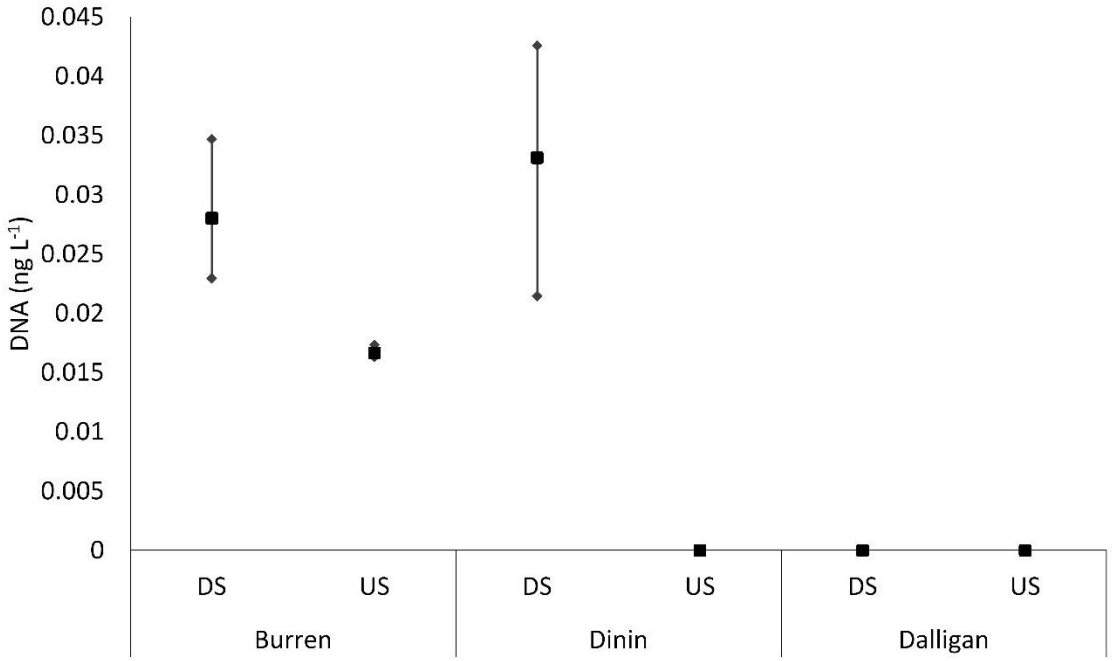

Figure 2. Graph showing the mean and range (maximum and minimum) of *S. salar* eDNA concentrations (ng L^{-1}) at each location (downstream (DS) or upstream (US) of the river obstacle) within each river sampled.