

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

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1 Abstract

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

13 Keywords: eDNA, conservation, monitoring, qPCR, minor groove binding (MGB) probe, river
14 obstacle, river barrier, field validation.

15

16 1. Introduction

17 The Atlantic salmon, (*Salmo salar* L.), is of ecological, cultural and economic importance. As
18 a result, this species has been the subject of intense exploitation ranging from commercial
19 fisheries, recreational fishing and intensive aquaculture (Morton, Ariza, Halliday, & Pita, 2016;

20 Piccolo & Orlikowska, 2012). Although *S. salar* is protected under Annex II and Annex V of
21 the EU Habitats Directive, and efforts to reduce fishing pressure and restore freshwater habitats
22 have been implemented, this once abundant species has continued to decline (Chaput, 2012;
23 Friedland et al., 2009). Numerous factors including recruitment failure at sea (Chaput, 2012;
24 Friedland et al., 2009), obstacles to migration in freshwater (Thorstad, Økland, Aarestrup, &
25 Heggberget, 2008) and pollution from agricultural, industrial and urban sources (Hendry,
26 Cragg-Hine, O’Grady, Sambrook, & Stephen, 2003) have contributed to the deterioration of *S.*
27 *salar* populations. Furthermore, the species is used for intensive aquaculture outside its native
28 range. Large escapes of *S. salar* happen with regularity in these areas, causing concerns about
29 the species’ invasive potential (Fisher, Volpe, & Fisher, 2014; Piccolo & Orlikowska, 2012).
30 To adequately address these issues, and to achieve the conservation objectives of the species,
31 it is vital to have knowledge on its distribution. At present, *S. salar* monitoring involves
32 electrofishing surveys, the placement of fish counters or traps, rod catch data provided by
33 anglers and redd counts (The Standing Scientific Committee on Salmon, 2016). These surveys
34 can be expensive, labour intensive and also potentially harmful to the fish (Snyder, 2004).
35 Clearly, there is a need for an effective, efficient and non-invasive sampling method to monitor
36 the species. To this end, environmental DNA (eDNA) analysis may provide an alternative
37 sampling strategy for monitoring the distribution of *S. salar* for management and conservation
38 purposes. Environmental DNA is the collective term for DNA present freely in the environment
39 which has been shed by organisms (in the form of mucus, faeces, gametes or blood, for
40 example), and can be extracted (Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012; Thomsen
41 & Willerslev, 2015). It has been shown to be an effective method for detecting species in
42 freshwater (Clusa, Ardura, Fernández, Roca, & García-Vázquez, 2017; Gustavson et al., 2015),
43 marine (Gargan et al., 2017) and terrestrial (Willerslev, 2003) environments.

44 Recent studies have developed and deployed specific primers for the detection of *S. salar* in
45 eDNA water samples. A study by Clusa, Ardura, Fernández, Roca, & García-Vázquez (2017),
46 for example, developed *S. salar*-specific primers using the 16S ribosomal DNA (rDNA)
47 region. These authors successfully identified *S. salar* in their eDNA samples using PCR-RFLP
48 (Polymerase chain reaction- restriction fragment length polymorphism). Alternatively, Dalvin,
49 Glover, Sørvik, Seliussen and Taggart (2010) utilised the mitochondrial DNA (mtDNA)
50 cytochrome c oxidase (COI) gene for their primer development, followed by traditional PCR
51 analysis. While the COI primers in this study were successful in amplifying DNA from tissue
52 samples (both fresh and degraded) the authors were unable to detect *S. salar* DNA in their

53 eDNA samples (Dalvin et al., 2010). The assay presented here provides an improvement on
54 these studies. As well as developing species-specific primers with the COI gene, the present
55 assay incorporates an additional species-specific minor groove binding (MGB) probe which
56 allows the eDNA sample to be analysed in quantitative PCR (qPCR). Furthermore, the MGB
57 probe allows for additional sensitivity and specificity of the assay, as three sequences as
58 opposed to two are checked against the target template DNA (Herder et al., 2014).

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

63 **2. Methods**

64 **2.1 eDNA qPCR assay development**

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

78 **2.2. Study area and field validation of *S. salar***

79 Three rivers located in the south of Ireland were selected for field validation of the eDNA
80 assay: the Dalligan, Dinin and Burren rivers (Table 1, Figure 1). Each of these rivers contains
81 an obstacle or barrier, which has the potential to prevent or delay the migration of *S. salar*.
82 Electrofishing was carried out by Inland Fisheries Ireland upstream and downstream of each
83 obstacle in July 2017 to verify the presence or absence of *S. salar* at each site. Environmental
84 DNA samples were collected on the same day that the electrofishing was carried out.

85 **2.3. eDNA collection, filtering and extraction**

86 Environmental DNA samples were collected from each river in sterilized 2L containers, and
87 filtered in the field using a peristaltic pump. Three replicate eDNA samples were collected both
88 upstream and downstream of each river obstacle. One negative field control per location
89 (upstream and downstream) consisting of ddH₂O was also filtered, resulting in a total number
90 of six eDNA samples and two field controls collected per river. Environmental DNA was
91 collected on 47 mm glass microfiber filters (1.5 μm) and placed into 2.0 mL Eppendorf tubes
92 prior to being frozen at -20° C. All work with eDNA was carried out in a dedicated Low Copy
93 DNA laboratory to reduce contamination risk. Environmental DNA was extracted using a
94 modified version of the CTAB (cetyltrimethylammonium bromide) protocol (Möller,
95 Bahnweg, Sandermann, & Geiger, 1992). One-half of a glass microfiber filter was placed into
96 a new 2.0 mL Eppendorf tube, to which 750 μL of CTAB buffer (100 mM Tris-HCL, 20 mM
97 EDTA, 1.4 M NaCl, 0.2%, 2% CTAB), and 7 μL of Proteinase K (20 mg mL⁻¹) was added.
98 Samples were vortexed for 10 seconds and incubated at 56° C for 2 hours, after which 750 μL
99 of Phenol/Chloroform/Isoamyl Alcohol (25:25:1 v/v) was added. Samples were manually
100 mixed for 15 seconds and centrifuged (11,000 x g, 20 min). The aqueous phase was transferred
101 to a new tube containing 750 μL of Chloroform/Isoamyl Alcohol (24:1 v/v), the manual mixing
102 and centrifugation steps were repeated, and the aqueous phase was transferred to a new tube.
103 The eDNA was then precipitated by adding one volume of isopropanol alcohol to the aqueous
104 phase and incubating the mixture at -20° C for 1 hour, and then centrifuged (11,000 x g, 20
105 min). The pellets were washed with 750 μL of 70% ethanol and centrifuged (11,000 x g, 5
106 min). The ethanol was carefully removed, and the pellets dried in a heating block (50° C, 5
107 min) before resuspending the eDNA in molecular-grade water.

108 **2.4. eDNA assay deployment**

109 Environmental DNA concentrations were determined by qPCR using an Applied Biosystems
110 ViiA™ 7 (Life Technologies, Inc., Applied Biosystems, Foster City, CA) quantitative
111 thermocycler. The qPCR reaction was conducted in a final reaction volume of 30μL, comprised
112 of 15 μL of TaqMan® Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems,
113 Foster City, CA), 3 μL of each primer (final concentration of 2 μM), probe (final concentration
114 of 2 μM), DNA template (3 μL) and ddH₂O. Warm-up conditions of 50°C for 2 min and 95°C
115 for 10 min, followed by 40 cycles between 95°C for 15 s and 60°C for 1 min were used for the
116 qPCR run. DNA extracted from *S. salar* tissue (quantified with NanoDrop®-1000, Thermo

117 Scientific, Wilmington, DE) was used to generate the standard curve using seven 10:1 serial
118 dilutions. Concentrations for the serial dilution ranged from 3ng/ μ L to 3×10^{-6} ng/ μ L. The
119 eDNA field samples were run on two separate 96-well clear qPCR plates. Each plate had 3 no-
120 template controls (NTCs) to ensure no contamination occurred during the preparation of the
121 qPCR plate. Individual standard curves were generated for each qPCR plate ($y = -3.22x +$
122 19.968 , efficiency = 100.018% (1) and $y = -3.25x + 20.091$, efficiency = 103.101% (2)). All
123 standard curve samples, field samples and controls were quantified in triplicate (three technical
124 replicates). A positive detection was defined as being within the range of the standard curve,
125 and when at least 2 out of the 3 technical replicates contained amplifiable DNA with Cq
126 differences not exceeding 0.5. If the difference between 1 out of 3 technical replicates exceeded
127 0.5Cq, this technical replicate was excluded from the study. However, if the Cq value of 2 out
128 of 3 technical replicates differed by more than 0.5Cq, the entire sample was excluded from
129 further study. As *S. trutta* was present in all rivers, both upstream and downstream of the
130 obstacles (Table 1), this species was used as a positive field control to test for the presence of
131 amplifiable DNA in sites where no *S. salar* was recorded during electrofishing surveys. The *S.*
132 *trutta* assay from previously published work (Gustavson et al., 2015) was used on eDNA
133 samples from above the bridge apron in the Dinin river, and above and below the weir in the
134 Dalligan river. Three replicates per location with one technical replicate were used for this
135 analysis.

136 **3. Results and Discussion**

137 The present assay was successful in detecting *S. salar* DNA *in silico*, *in vitro* and *in situ*. The
138 assay did not amplify the DNA of closely related species (*S. trutta*) or any other species
139 included in the specificity test. The lowest detected eDNA concentration within the range of
140 the standard curve was 0.016 ng L⁻¹ at Cq 34.5 (average over 3 technical replicates, standard
141 deviation 0.0015 ng L⁻¹). For the purposes of analysis, one technical replicate from the 1:7
142 serial dilution was disregarded (equation 1), and the entire 1:7 dilution for the standard curve
143 (equation 2) was disregarded because differences in Cq values between either one or more
144 technical replicates in these samples exceeded 0.5. For the remainder of the samples, however,
145 the standard deviation between technical replicate Cq values ranged from 0.011 to 0.303.

146 The results of the eDNA analysis mirrored what was observed in the electrofishing surveys. At
147 each site where the presence of *S. salar* was confirmed by electrofishing, its presence was
148 confirmed by eDNA analysis (Table 2, Figure 2). At sites where *S. salar* was not detected by

149 electrofishing, a negative result was also obtained in the eDNA samples when assessed with
150 the *S. salar* assay (Table 2, Figure 2). However, detectable eDNA was confirmed at all sites
151 including the sites where no *S. salar* DNA was detected, as amplification occurred when the
152 same samples were run in qPCR with the *S. trutta* assay. No DNA was amplified in any of the
153 NTCs or negative field controls.

154 The assay successfully confirmed either the presence or absence of *S. salar* in each sampling
155 location, demonstrating the potential future use of this assay for detecting the species without
156 traditional sampling methods. Furthermore, this eDNA assay would be particularly valuable
157 for monitoring *S. salar* year-round. Traditional sampling methods are typically carried out
158 during specific times of the year. For example, redd counts are only possible during the
159 spawning period, and electrofishing surveys are typically restricted to the summer months
160 when water levels are low, and fish are not migrating. While fish counters and traps can provide
161 year-round records of *S. salar* movements, the structures themselves can act as obstacles to the
162 movement of other, non-salmonid fish. For example, resistivity fish counters are typically
163 placed on sloping weir-like structures (Lucas & Baras, 2000) which have been shown to impede
164 the movement of lamprey *Lampetra fluviatilis* L. (Lucas, Bubb, Jang, Ha, & Masters, 2009;
165 Russon, Kemp, & Lucas, 2011) and barbel *Barbus barbus* L. (Lucas & Frear, 1997).

166 While this study clearly demonstrates the value of eDNA as a tool for monitoring the impact
167 of river obstacles on *S. salar*, it could be applied in numerous different contexts including
168 monitoring *S. salar* escapes from fish farms outside the native range. In addition, the use of
169 eDNA as a monitoring tool could largely reduce the spread of invasive alien species, since
170 eDNA sampling must be carried out with minimum equipment that must be sterilised to avoid
171 DNA contamination (Herder et al., 2014).

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

177 **Acknowledgements**

178 This project was funded by the Atlantic Salmon Trust (Salmo Slime project) with additional
179 support from the Irish Environmental Protection Agency (Reconnect project). The authors

180 would like to thank Inland Fisheries Ireland for carrying out all the electrofishing surveys
181 which supported this research.

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245 pleistocene sediments. *Science*, 300, 791–795.

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	<i>S. salar</i> Downstream	<i>S. salar</i> Upstream	<i>S. trutta</i> Downstream	<i>S. trutta</i> 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 (\pm SD) are given for each location (upstream or downstream of the river obstacle).

River	Location	<i>S. salar</i> 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 \pm 0.31	0.028 \pm 0.006
	Upstream	Yes	1	34.468	0.017	
			2	34.553	0.016	
3			34.549	0.016		
				34.523 \pm 0.05	0.017 \pm 0.001	
Dinin	Downstream	Yes	1	32.616	0.043	
			2	32.861	0.035	
			3	33.569	0.021	
					33.015 \pm 0.5	0.033 \pm 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	

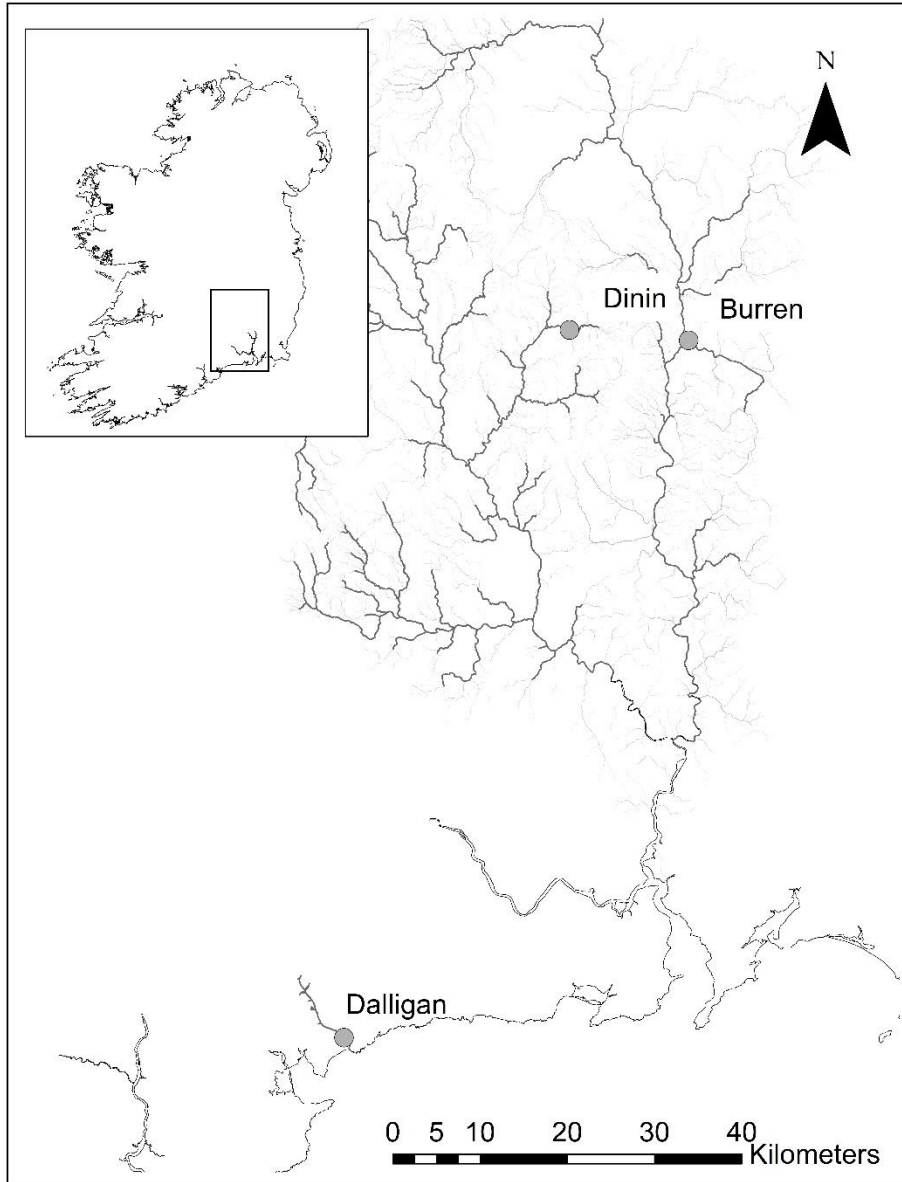


Figure 1. Map showing the locations of the sampling sites in this study.

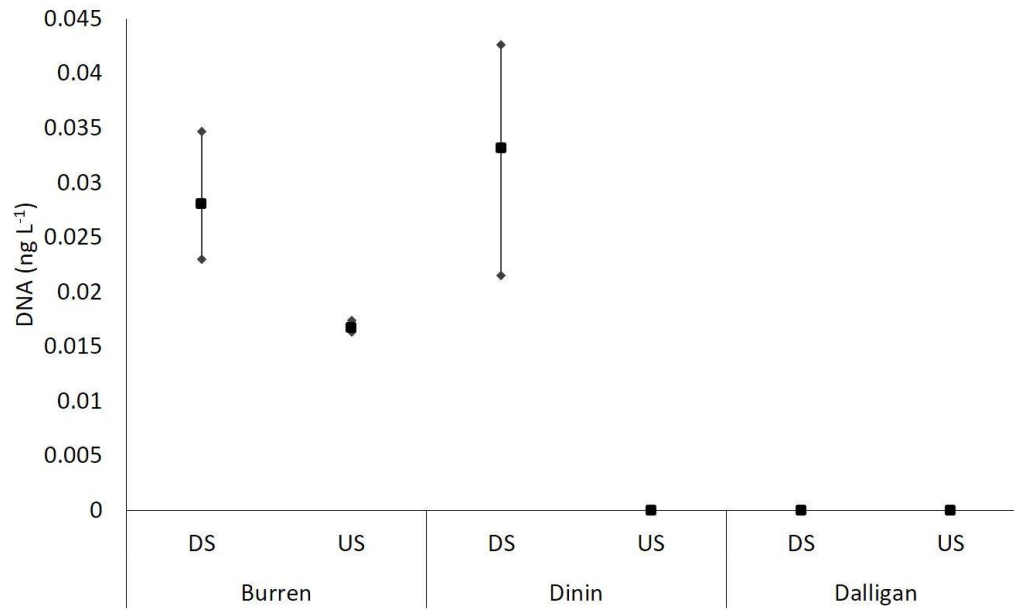


Figure 2. Graph showing the mean and range (maximum and minimum) of *S. salar* eDNA concentrations (ng L⁻¹) at each location (downstream (DS) or upstream (US) of the river obstacle) within each river sampled.