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Development of a real-time PCR assay for the identification of *Gyrodactylus* parasites infecting salmonids in northern Europe

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ABSTRACT: *Gyrodactylus salaris* is a monogenean freshwater parasite that causes high mortality in wild Atlantic salmon, and a number of countries employ monitoring programmes for its presence. A TaqMan®-MGB (minor groove binding) probe real-time multiplex assay targeting the internal transcribed spacer ribosomal DNA (ITS rDNA) was developed to simultaneously identify *G. salaris*/*G. thymalli* and 2 other commonly occurring *Gyrodactylus* species infecting salmonids in northern Europe: *G. derjavinoides* and *G. truttae*. In addition, a *Gyrodactylus* genus-level assay was developed to assess parasite DNA quality. The species-specific real-time PCR method correctly identified target species from a wide geographical range and from a number of salmonid hosts. It did not amplify *G. lucii* or *G. teuchis*. These species were successfully amplified using the *Gyrodactylus* genus real-time assay. The species-specific real-time assay proved to be significantly faster than the currently employed molecular screening method of ITS rDNA PCR amplification followed by restriction fragment length polymorphism analyses (RFLP). However, as with ITS RFLP, the real-time method did not distinguish between *G. salaris* and the non-pathogenic *G. thymalli*, its principle advantage being a significant reduction in time to achieve an initial diagnostic screen before the employment of more in-depth analyses for those specimens giving a positive *G. salaris*/*G. thymalli* real-time result.

KEY WORDS: *Gyrodactylus salaris* · Diagnostic · TaqMan-MGB real-time PCR

INTRODUCTION

Gyrodactylus salaris is a monogenean ectoparasite of freshwater salmonids. To date it has only been reported within Europe (Malmberg 1993). It is highly virulent towards Atlantic salmon *Salmo salar* L., eastern Atlantic salmon strains. Its direct lifecycle (Jansen & Bakke 1991, Cable & Harris 2002) and the apparent inability of Atlantic salmon to control *G. salaris* infection results in population explosions of the parasite on the host. Since its introduction to Norway in the late 1970s, *G. salaris* has caused the decimation of a number of important salmon rivers (Johnsen et al. 1999, Peeler et al. 2006).

Gyrodactylus salaris is included on list III of the Office International des Épizooties (OIE) as a notifiable pathogen. In Europe it is not listed under Council Directive 2006/88/EC (Council of the European Union 2006), but is covered by some countries under 'additional guarantees' set out in Commission Decision 2004/453/EC. Even though there is no obligation to monitor for the presence of *G. salaris* in areas declared free of the parasite, many countries still maintain monitoring programmes with the aim of an early warning of its introduction so that management and eradication programmes can be implemented and damage to wild salmon populations minimised.

The classical method of *Gyrodactylus* species identification is through morphological examination of the opisthaptor (attachment organ) hard parts. Differences between some species can be quite small and thus a high level of expertise is required. PCR amplification of the internal transcribed spacer ribosomal DNA (ITS rDNA) followed by analysis of restriction fragment length polymorphism (RFLP) patterns (Cunningham 1997) has been used for a number of years in monitoring programmes for *G. salaris* in Scotland.

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The taxonomy of *Gyrodactylus salaris* is complicated by the description of *G. thymalli*, a parasite of grayling *Thymallus thymallus*, which is not pathogenic to Atlantic salmon (Bakke et al. 2002, Sterud et al. 2002). *G. salaris* is morphologically very similar to *G. thymalli*. *G. thymalli* and most strains of *G. salaris* also share identical ITS rDNA sequence, so that ITS RFLP cannot be used to differentiate between them. Some authors (Hansen et al. 2006, Kuusela et al. 2007) consider *G. thymalli* synonymous with *G. salaris*, although this has not been formally accepted. In addition, gyrodactylids identified as *G. salaris* which do not display pathogenicity towards Atlantic salmon have been found on charr and rainbow trout (Lindenstrøm et al. 2003, Jørgensen et al. 2007, Olstad et al. 2007, Robertsen et al. 2007). These *G. salaris* strains differ in 1 to 3 nucleotide positions in their ITS rDNA compared to pathogenic forms. Phylogenetic analysis of mitochondrial cytochrome c oxidase subunit I (COI) sequences has been used to discriminate between *G. thymalli* and *G. salaris* populations (Hansen et al. 2003, Meinilä et al. 2004). However, no definitive nucleotide difference in the COI region exists which identifies all *G. salaris* or all *G. thymalli* only, and new COI variants, especially for *G. thymalli* populations, are routinely discovered (Hansen et al. 2007). Therefore, the development of a simple, robust and relatively quick diagnostic test based on COI sequence discrimination is difficult. As such, the most robust and efficient approach to ensure rapid identification of *G. salaris* using currently available markers is to apply a rapid primary screen to identify all *G. salaris*/*G. thymalli* strains, followed by targeted COI sequencing to provide a more definitive identification. The objective of the current work was to enhance this initial screening process through the development of faster, more efficient molecular tests that are capable of dealing with high numbers of parasites.

Real-time PCR is used increasingly in diagnostic assays (Cavender et al. 2004, Corbeil et al. 2006, Snow et al. 2006, Jansson et al. 2008). It can offer higher sensitivity and, depending on the chemistry used, higher specificity through the use of a probe alongside the amplification primers. Although the actual real-time reaction is more costly than traditional PCR, this is offset by savings in subsequent costs and time, as post PCR processing steps are no longer required for detection of the PCR product and in some cases determination of the pathogen species, e.g. as when using RFLP. As such it is also very amenable to automation. Due to the last 2 factors, a real-time PCR assay for monitoring and detection of *Gyrodactylus salaris*/*G. thymalli* was considered a useful tool to develop, both for routine monitoring, and in particular in the event of a *G. salaris* outbreak where high parasite numbers may need to be processed.

MATERIALS AND METHODS

Rationale of approach. The objective of the current work was to develop a real-time PCR assay to replace ITS rDNA RFLP methodology in screening for potential *Gyrodactylus salaris* parasites. This should allow a significant reduction in time to obtain an initial diagnostic result. As with ITS/RFLP, the assay does not differentiate between *G. salaris* and *G. thymalli*.

Given that in some regions most or all specimens analysed may not be *Gyrodactylus salaris*/*G. thymalli*, we decided to develop additional assays for other commonly occurring *Gyrodactylus* species found on salmonids, viz. *G. derjavinoides* and *G. truttae*, and to use the 3 assays in a multiplex format. In this way, a positive result for *G. derjavinoides* or *G. truttae* would ensure that a negative *G. salaris*/*G. thymalli* result was not related to problems with the specimen such as DNA extraction, or with the *G. salaris*/*G. thymalli* assay. In addition, the specificity of the *G. derjavinoides* and *G. truttae* assays increase the overall robustness of the multiplex assay as opposed to running a generic *Gyrodactylus* assay, as a positive generic/negative *G. salaris*/*G. thymalli* result would not exclude the possibility that *G. salaris* was still present, but the specific assay not working. A generic assay for *Gyrodactylus* species was developed and tested alongside species-specific assays. A flow chart showing the proposed use of the real-time assay in *Gyrodactylus* diagnostics is given in Fig. 1.

Reference *Gyrodactylus* material for real-time PCR development: DNA extraction and species verification. *G. salaris* originating from the River Lærdalselva, Norway, were obtained from Dr. T. A. Mo (National Veterinary Institute Oslo). *G. derjavinoides* and *G. truttae* parasites were obtained from Scottish farmed and freshwater sites during routine sampling. DNA was released from parasites following tissue lysis in 10 µl of lysis buffer, without further purification, as described in Cunningham et al. (2001), but substituting NP40 with its chemical equivalent IGEPAL ca-630 (Sigma). The *Gyrodactylus* specimens used to generate crude lysates were identified molecularly by PCR amplification of their ITS rDNA followed by RFLP analysis with the restriction enzyme *Hae*III (Cunningham et al. 2001).

Testing for inhibition due to lysate in real-time PCR reactions. To determine if the parasite lysate caused inhibition of the real-time reaction, amplification of Taqman® exogenous internal positive controls (IPCs; Applied Biosystems) was carried out in triplicate in the presence of undiluted and 10^{-1}- to 10^{-4}-fold serial dilutions of *Gyrodactylus salaris* lysate. The reaction consisted of 1× TaqMan Universal PCR Master mix (with uracil N-glycosylase [UNG]; Applied Biosystems), 1× Exo IPC Mix (containing primers and probe for exoge-

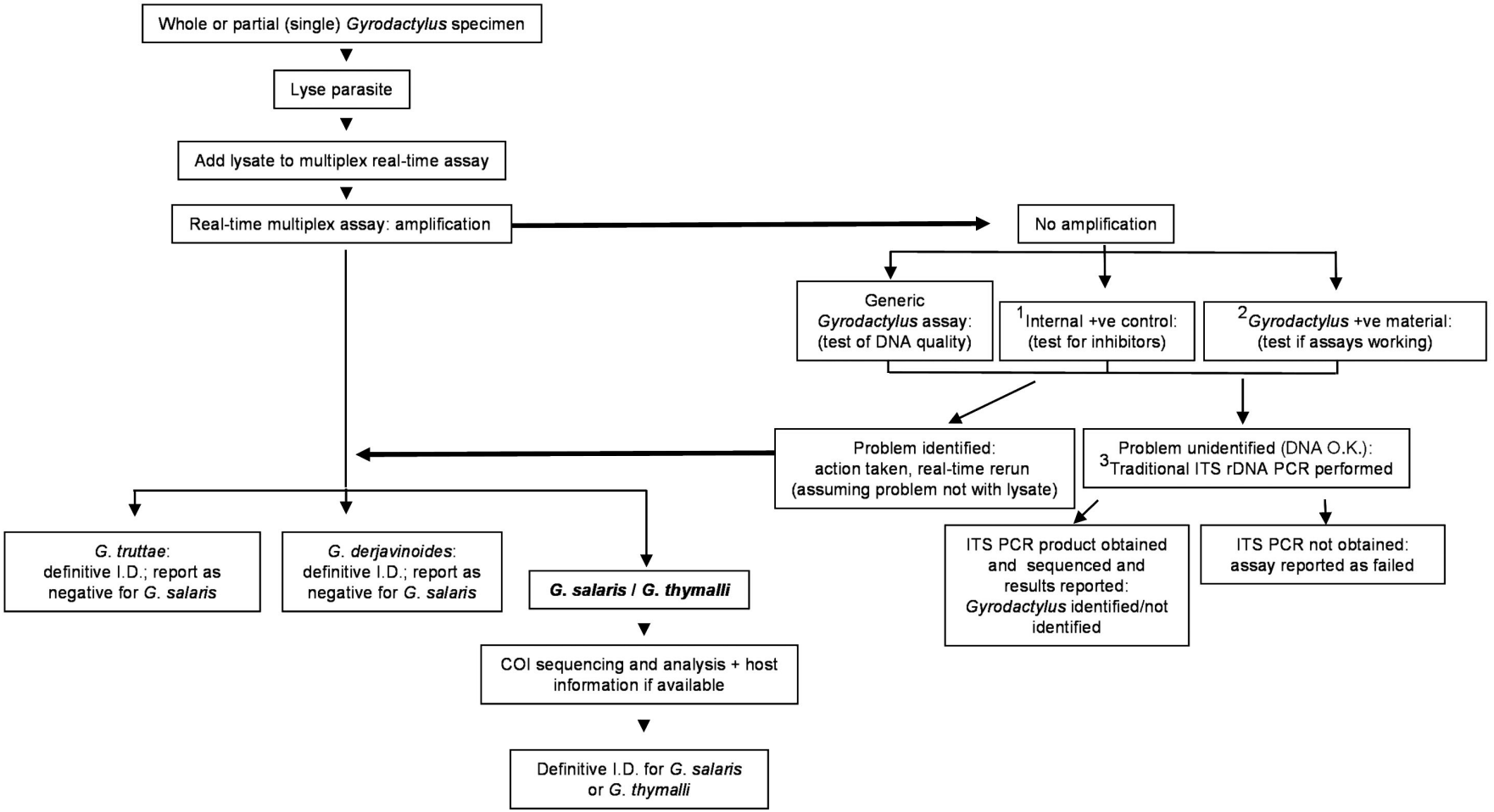

Fig. 1. *Gyrodactylus* diagnostic workflow. Proposed use of the TaqMan-MGB® real-time assay for *Gyrodactylus* diagnostics. The diagram does not include morphological steps which might be included in an overall diagnostic approach but instead focuses on molecular identification of the parasite. 1: Synthetic target DNA and complementary real-time assay supplied by commercial company. 2: High dilution characterised plasmid/PCR product or synthetically produced product incorporating *Gyrodactylus* real-time primer and probe sequences. 3: Traditional PCR is highly conserved and should amplify *Gyrodactylus* (and related genera) in many cases. COI: cytochrome c oxidase I; ITS rDNA: internal transcribed spacer ribosomal DNA

nous IPC amplification) and 1× Exo IPC DNA (Applied Biosystems) in addition to 1 µl of *G. salaris* lysate or dilutions of lysate. Cycle threshold (Ct) values for the IPCs in reactions containing *G. salaris* lysate were compared to 3 reactions without *G. salaris* lysate. In addition, undiluted and 10^{-1}- to 10^{-4}-fold serial dilutions of the lysis buffer alone were added to IPC real-time PCR reactions in triplicate to determine if the buffer constituents rather than the *Gyrodactylus* tissue might be responsible for any observed inhibition. Ct values for IPC amplification in the presence of lysis buffer were compared to the IPC Ct values from reactions without lysis buffer. The Ct values were compared using Student's *t*-test.

Probe and primer design. ITS rDNA sequences from *Gyrodactylus* species belonging to the subgenus *G. Limnonephrotus*, *G. wageneri* group, which includes *G. salaris* and other commonly found salmonid parasites in European freshwaters such as *G. derjavinoides*, *G. truttae* and *G. teuchis*, as well as *Gyrodactylus* species belonging to other subgenera (Malmberg 1970, Ziętara et al. 2002, Matejusová et al. 2003), were downloaded from the public databases and aligned using CLUSTALW software (Thompson et al. 1994). TaqMan real-time PCR primers and probes were designed by eye, following recommended guidelines (Applied Biosystems) as far as possible, i.e. amplicon between 50 and 150 nucleotides in length, primers with a melting temperature (Tm) between 58 and 60°C, >2°C difference in Tm between primers, 20 to 80% GC content, maximum of 2 G/C's in last 5 bases at 3′ end, probe; 10°C higher than primer Tm, 20 to 80% GC content, no G at 5′ end, <4 contiguous G's, must have more C's than G's. Assays were designed for *G. salaris*, *G. derjavinoides* and *G. truttae*, choosing regions which showed interspecific variation in both primer and probe sequences, to increase specificity, but which were conserved intraspecifically. A generic assay for *Gyrodactylus* species was designed based on conserved sequences in the 5.8S rDNA of *Gyrodactylus* species from different subgenera. Primers and probes were used to search public databases for homologous sequences, using the megablast algorithm (Altschul et al. 1997) and parameters adjusted for short nucleotide sequences, to check their specificity for the target organisms. Oligo Analyzer (www.cmbn.no/tonjum/biotools-free-software.html) was used to test for likelihood of primer/probe dimer formation. Primers and TaqMan® MGB (minor groove binding) probes (detailed in Table 1) were obtained from Invitrogen and Applied Biosystems (Applera), respectively.

Real-time PCR reactions: single target template. The multiplex real-time PCR consisted of 3 sets of primers and probes, targeting *Gyrodactylus salaris*/ *G. thymalli*, *G. derjavinoides* and *G. truttae*, respectively. The real-time PCR assay was performed on an ABI 7000 Sequence Detection System (Applied Biosystems). One microlitre of lysate (from a single species: *G. salaris*, *G. derjavinoides*, *G. truttae*, *G. teuchis* or *G. lucii*) was added to the following mix contained in individual wells of a 96-well optical plate (Applied Biosystems): 1× TaqMan Universal PCR Master mix (with UNG; Applied Biosystems), 0.9 µM of each forward and reverse primer and 0.25 µM of each probe and dH_2O (Sigma) to a final volume of 20 µl. The cycling conditions were 50°C for 2 min, 95°C for 10 min followed by 35 cycles of 95°C for 15 s and 60°C for 1 min. The threshold level was set at 0.2, and the baseline was determined automatically by ABI 7000 software.

The real-time PCR assays for *Gyrodactylus salaris*/ *G. thymalli*, *G. derjavinoides* and *G. truttae*, which were used in the multiplex, were also run individually to test their efficiency as singleplexes. Reactions were as for multiplex reactions but containing primers and probe for single assays only. Cycling conditions were as for the multiplex reaction above.

The generic real-time assay for *Gyrodactylus* species was performed as described above for multiplex assays but using the genus-level forward and reverse primers, and TaqMan probe, and using 0.5 µl of lysate. Cycling conditions were as for the multiplex assays above.

Assay efficiency and detection limits. The *Gyrodactylus* spp. crude lysates were diluted with dH_2O (Sigma) in a 10-fold series, ranging from 10^{-1} to 10^{-11}. Real-time PCR reactions were performed in triplicate, as described above, for each dilution of parasite lysate (*G. salaris*, *G. derjavinoides*, *G. truttae*). Ct values for the reactions were plotted against $-\log_{10}$ dilution, and the slope (m) of the regression line was calculated. The % amplification efficiency was calculated as $100 \times (10^{1/m} - 1)$ (Rutledge & Cote 2003). An m value of –3.3 represents 100% efficiency. Detection limits were determined based on the highest lysate dilution giving a positive result in all of its triplicate real-time reactions. Traditional ITS PCR and *Hae*III RFLP was also performed in duplicate on each of the dilutions to compare its limit of detection to those obtained for real-time PCR. The ITS PCR and *Hae*III digest were performed as described in Cunningham (1997) and Cunningham et al. (2001).

Specificity testing. The multiplex assay was tested against its individual target species (*Gyrodactylus salaris*/*G. thymalli G. derjavinoides*, *G. truttae*) and non-target species (*G. teuchis*, *G. lucii*) from widespread geographic areas. These specimens had previously been characterised using morphology, statistical classifiers and ITS rDNA RFLP as part of a comparative proficiency test of established diagnostic methods (Shinn et al. 2010) (Table 2). All specimens had been characterised by each method and the ITS rDNA and COI mtDNA sequenced from a number of them.

Table 1. Primers and probes used in TaqMan-MGB® real-time PCR to identify *Gyrodactylus* species. F: forward; R: reverse

Probe	Probe sequence (5′–3′)	Primer sequence (5′–3′)	DNA strand sequenced	PCR product size (bp)
Gsal2	FAM-TCTTATTAACCAGTTCTGC	F: CGATCGTCACTCGGAATCG R: GGTGGCGCACCTATTCTACA	Reverse	60
Gder2	VIC-CTGCTCAGAACTCATCTC	F: TTGGTGCCCCATGATGGT R: CGCACCTATTCAGCTAGTCTTAATACA	Reverse	70
Gtrutt2	NED-CCTTCGTTGTGCCCC	F: TCGACGGACTCGATCGTTTC R: CGCACCTGTTCTACTATAGACGAAGT	Reverse	64
pGen	FAM-TTGCTGCACTCTTCATCG	F: TGGATCACTCGGCTCACG R: TGCGTTTCACATTGGTTAACACA	Reverse	64

The genus level assay was tested on a subset of the above samples including specimens from all species available.

Species level multiplex real-time PCR: multiple target template. The ability of the real-time multiplex assay to detect *Gyrodactylus salaris* in pooled lysates was determined using 1 µl of template from a 1:10, 1:20 and 1:100 *G. salaris*:*G. derjavinoides* lysate mix, the parasites having been initially lysed individually in 10 µl buffer as described previously.

RESULTS

Testing for inhibition in real-time PCR reactions

IPC amplification in reactions containing working concentration and serially diluted lysis buffer was not affected and did not differ significantly from IPC amplification in buffer-free controls. Addition of *Gyrodactylus* lysate also did not result in inhibition of IPC amplification. The average Ct value obtained for IPC amplification in a reaction mix without lysis buffer or *Gyrodactylus* lysate was 28.74 ± 0.04 (mean ± SE, n = 5). Average Ct values (n = 3) for IPC amplification in the presence of a working concentration of lysis buffer or *Gyrodactylus* lysate, or in the presence of serial dilutions of these (10^{-1}- to 10^{-4}-fold), ranged from 28.57 to 28.92.

Probe and primer design

BLAST analysis showed that some of the species-specific primers or probes gave a 100% match to a number of unrelated organisms; however, the primers and probe combinations used in each assay only gave a 100% match for those species targeted by the assay.

The generic assay primer and probe sequences showed 100% homology to the 5.8S rDNA of most *Gyrodactylus* species for which sequence data are available. A number of species, 16 in total, contained mismatches with the primers or probe nucleotide sequences. Of these, most mismatches could be accounted for by poor sequences, with only 3 species having reliably different nucleotides: *G. poeciliae* (AJ001844), *G. rogatensis* (AJ011411) and *Gyrodactylus* sp. (AY099508: from *Rhynichthis osculus*, Idaho, USA).

Assay efficiency and detection limits

The efficiencies of the multiplex and singleplex assays ranged from 93.1 to 100.5% and from 93.1 to 101.1%, respectively (Table 3).

Table 2. Parasite specimens used in testing of *Gyrodactylus* spp. multiplex and generic real-time assays

Parasite species	No. of parasites tested with multiplex assay (generic assay)	Host species	Country
G. salaris/*G. thymalli*	22 (8)	*Salmo salar, Oncorhynchus mykiss, Thymallus thymallus*	Norway[a], Finland[a], Denmark[a], England[b]
G. derjavinoides	100 (27)	*S. salar*, *S. trutta*, *O. mykiss*, *T. thymallus*	Scotland, England, N. Ireland, Denmark, Italy
G. truttae	42 (13)	*S. trutta*	N. Ireland, Scotland
G. lucii	9 (11)	*Esox lucius*	England
G. teuchis	2 (2)	*O. mykiss*	Italy

[a]Specimens identified as *G. salaris* following cytochrome c oxidase subunit I (COI) sequence analysis
[b]Specimens identified as *G. thymalli* following COI sequence analysis

Detection limits for the real-time assays and for traditional ITS PCR/RFLP analysis on the same *Gyrodactylus* lysates and dilutions thereof are given in Table 3. The *G. salaris*/*G. thymalli* real-time assay in multiplex and singleplex format identified *G. salaris* at 10- to 100-fold higher dilutions compared to traditional PCR/RFLP. The *G. derjavinoides* and *G. truttae* real-time assays in multiplex format showed the same level of sensitivity as traditional PCR/RFLP in their ability to identify the parasites, whereas the assays in singleplex format identified parasites at 10-fold higher dilutions.

Specificity testing

The real-time multiplex assay identified all specimens of *Gyrodactylus salaris*/*G. thymalli*, *G. derjavinoides* and *G. truttae* correctly, with most Ct values ranging from 16.34 to 21.28, and a single *G. salaris* specimen giving a Ct value of 27.15. As expected, the assay did not differentiate *G. salaris* from *G. thymalli*. The multiplex assay did not show amplification for the non-target species *G. lucii* and *G. teuchis*.

A subset of the specimens used for specificity testing above was used with the generic assay. The generic assay amplified all *Gyrodactylus* species, including those not amplified by the species-specific multiplex assay, with Ct values ranging from 16.11 to 20.52.

Species level multiplex real-time PCR: multiple target template

The Ct values for 1:10, 1:20 and 1:100 lysate mixes of *Gyrodactylus salaris*:*G. derjavinoides* were 20.69 ± 0.17:17.78 ± 0.12, 22.14 ± 0.12:17.80 ± 0.06, and 24.39 ± 0.11:17.60 ± 0.03 (n = 3), respectively.

DISCUSSION

Generic and species-specific real-time assays for *Gyrodactylus* parasites were developed during the current study. The generic assay was designed to amplify most members of the genus *Gyrodactylus* and successfully amplified all 6 species tested, although all were from the subgenus *G. Limnonephrotus*. The generic assay was designed against the ITS region (5.8S gene), as this represented the largest body of available sequence data. A small number of species showed nucleotide differences in their ITS rDNA when the assay's primer and probe sequences were compared to available sequences in the public database. More conserved regions may exist in the 18S and 28S rDNA, and these may be good targets for future generic assays. The generic assay can be used in conjunction with a species assay, or in a second PCR with specimens that had not amplified with species-specific assays, to help determine if negative results are due to a non-target species or to bad quality DNA or PCR inhibitors.

The multiplex real-time assay developed here identifies *Gyrodactylus salaris*/*G. thymalli*, *G. derjavinoides* and *G. truttae*. It does not distinguish *G. salaris* from the non-pathogenic *G. thymalli*, which currently requires COI sequence analysis to differentiate between them (although differences in the hard parts of the attachment organ can be used by experts in morphology). Based on the ITS rDNA region targeted, the *G. salaris* assay also does not differentiate between the Norwegian pathogenic strains and recently described *G. salaris* strains from Demark and Norway (Lindenstrøm et al. 2003, Jørgensen et al. 2007, Olstad et al. 2007, Robertsen et al. 2007), which show lower pathogenicity to salmon. These strains have a low number of nucleotide differences in their ITS rDNA, and some

Table 3. PCR efficiency and detection limits for real-time assays and traditional PCR used to identify *Gyrodactylus* species. % efficiency = $100 \times (10^{1/m} - 1)$, where m = slope

Probe	Assay	R^2	*y*-intercept	Slope	PCR efficiency (%)	Limit of detection (dilution)	
						Real-time PCR	Traditional PCR/RFLP
Gsal2	Multiplex	0.997	17.90	3.43	95.7	10^{-4}	10^{-2} [a]
Gder2	Multiplex	0.997	17.17	3.50	93.1	10^{-3}	10^{-3} [a]
Gtrutt2	Multiplex	0.989	18.35	3.31	100.5	10^{-3}	10^{-3} [a]
Gsal2	Singleplex	0.998	17.68	3.50	93.1	10^{-4}	10^{-2} [a]
Gder2	Singleplex	0.998	18.06	3.37	98.1	10^{-4}	10^{-3} [a]
Gtrutt2	Singleplex	0.997	18.00	3.30	101.1	10^{-4}	10^{-3} [a]
Genus	Singleplex	0.975[b]	16.29[b]	3.46[b]	94.5 (91.8 to 97.4)[c]	10^{-6} to 10^{-7}	–

[a]Faint PCR products were observed at 10-fold higher dilution, but restriction fragment length polymorphism analysis (RFLP) results were too faint to determine species

[b]R^2, *y*-intercept and slope values for combined data from efficiency curves using *G. salaris* (Gs), *G. derjavinoides* (Gd) and *G. truttae* (Gtr) as template

[c]PCR efficiency (%) for the generic *Gyrodactylus* assay using combined data, and in parentheses the efficiency (%) range for the assay using Gs, Gd and Gtr lysates as template

have been differentiated based on RFLP patterns (Lindenstrøm et al. 2003, Kania et al. 2007). In theory, real-time assays could be designed based on these few nucleotide differences. However, the stability of the 'non-pathogenic' state in these strains, and under different environmental conditions, has not been investigated and as such an assay which identifies all *G. salaris* strains is more desirable when monitoring for initial introductions of the parasite. Additional assays for specific identification of non-pathogenic strains may be useful in regions where *G. salaris* is endemic and different strains exist.

The *Gyrodactylus salaris*/*G. thymalli* real-time multiplex assay provided a 10-fold lower level of detection compared to traditional PCR, and this may improve diagnostics in some cases where specimen DNA is degraded. Lower detection limits also mean that low levels of contaminating *G. salaris*/*G. thymalli* DNA can be detected more easily. Using the multiplex format, it would be easier to distinguish contamination, as the Ct values obtained for the true species should be considerably lower than the contaminant in most cases.

The detection limits of the *Gyrodactylus derjavinoides*/*G. truttae* real-time assays in the multiplex format were the same as those for traditional PCR, while detection limits for the assays in singleplex format were 10-fold lower, indicating that there may be some interaction between multiplexed primers and probes which reduces the individual efficiencies.

With respect to identification of individual *Gyrodactylus* parasites, higher detection limits are not as important an issue as for single-celled organisms such as viruses, bacteria or protozoa, since it involves the identification of a parasite known to be already present rather than detection of potentially low numbers of 'unseen' microscopic organisms.

In certain situations it may be desirable to pool *Gyrodactylus* DNA, as this would allow more parasites to be analysed per real-time plate, thereby reducing time and cost of screening. With respect to pooled parasites, *G. salaris* was detectable well within the 35 cycles used when diluted 1:10 to 1:100 with *G. derjavinoides* DNA. Currently, sequencing of the COI mtDNA is required to confirm a positive *G. salaris* result, as both the ITS RFLP and the real-time assay developed here do not differentiate *G. salaris* from *G. thymalli*. Although it appears to preferentially amplify *G. salaris*/*G. thymalli* DNA, the COI PCR (Hansen et al. 2003) has not been checked extensively against other parasites to test for specificity. In addition, the sensitivity of the COI PCR has not been tested. Pooling parasites prior to lysis, though faster for initial analysis, might result in delays obtaining subsequent sequence data for *G. salaris*/*G. thymalli* if present in the pool. Therefore, the proposed approach for analysing pooled samples is to maintain the lysates separately and pool aliquots of these, at least until such time as the specificity and sensitivity of the COI PCR has been verified.

If parasites were pooled before lysing, then depending on the final lysate volume, higher DNA concentrations for certain species might reduce detection limits for species in lower numbers, so the real-time assay would have to be re-evaluated for this approach.

The real benefit of the real-time PCR presented here is in the reduction in time to obtain a preliminary diagnosis, and the reduced time required of staff, the latter compensating for the higher reagent costs (which are variable, depending on supplier used and how many parasites are processed together). Compared to ITS PCR followed by RFLP and associated gel electrophoresis, the real-time PCR represents a single reaction with real-time provision of results. Once the parasites have been lysed to release DNA, identification can currently be obtained in approximately 80 min using the real-time assay as opposed to approximately 350 min (allowing 90 min for RFLP digestion) using ITS PCR/RFLP analysis (Cunningham 1997). The quoted times are for running of the reactions/gels only and do not include preparation time, which again would be considerably more for the ITSPCR/RFLP due to the additional steps in the methodology.

In summary, the species-specific real-time PCR presented here does not improve on the specificity of current molecular diagnostics in that it does not differentiate between *Gyrodactylus salaris* and *G. thymalli*. However, it allows quicker processing of samples and identification of potential *G. salaris* specimens, and as such represents a valuable tool in monitoring programmes for *G. salaris*. This is especially true for situations where large numbers of parasites require testing, such as following an outbreak or screening parasites to prove the absence of *G. salaris* after attempted eradication. Diagnostic labs are increasingly moving to real-time technology, and a real-time assay for *G. salaris* will allow *Gyrodactylus* cases to be run side by side with other pathogens on the same plate where space allows, again decreasing overall staff time. The real-time assay developed here is currently being used for *Gyrodactylus* identification at the Marine Laboratory, Marine Scotland, Aberdeen.

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