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Multi-centre testing and validation of current protocols for the identification of *Gyrodactylus salaris* (Monogenea)

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ABSTRACT

Despite routine screening requirements for the notifiable fish pathogen *Gyrodactylus salaris*, no standard operating procedure exists for its rapid identification and discrimination from other species of *Gyrodactylus*. This study assessed screening and identification efficiencies under real-world conditions for the most commonly employed identification methodologies: visual, morphometric and molecular analyses. Obtained data were used to design a best-practice processing and decision-making protocol allowing rapid specimen throughput and maximal classification accuracy. True specimen identities were established using a consensus from all three identification methods, coupled with the use of host and location information. The most experienced salmonid gyrodactylid expert correctly identified 95.1% of *G. salaris* specimens. Statistical methods of classification identified 66.7% of the *G. salaris*, demonstrating the need for much wider training. Molecular techniques (internal transcribed spacer region-restriction fragment length polymorphism (ITS-RFLP)/cytochrome *c* oxidase I (COI) sequencing) conducted in the diagnostic laboratory most experienced in the analysis of gyrodactylid material, identified 100% of the true *G. salaris* specimens. Taking into account causes of potential specimen loss, the probabilities of a specimen being accurately identified were 95%, 87% and 92% for visual, morphometric and molecular techniques, respectively, and the probabilities of correctly identifying a specimen of *G. salaris* by each method were 81%, 58% and 92%. Inter-analyst agreement for 189 gyrodactylids assessed by all three methods using Fleiss' Kappa suggested substantial agreement in identification between the methods. During routine surveillance periods when low numbers of specimens are analysed, we recommend that specimens be analysed using the ITS-RFLP approach followed by sequencing of specimens with a "*G. salaris*-like" (i.e. *G. salaris*, *Gyrodactylus thymalli*) banding pattern. During periods of suspected outbreaks, where a high volume of specimens is expected, we recommended that specimens be identified using visual identification, as the fastest processing method, to select "*G. salaris*-like" specimens, which are subsequently identified by molecular-based techniques.

1. Introduction

Identification of parasite pathogens to species level can be a complex and time consuming task. In the face of a potential disease outbreak, the need for identification may exceed the capacity to deal with the number of specimens entering the identification pipeline. Recent evidence demonstrates that the translocation of fish across national borders has increased the rate of introduction of exotic pathogens into indigenous fish stocks with serious economic consequences. Of 14 fish metazoan parasites recently reported to have been introduced into the United Kingdom (UK),

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10 are already fully established, and this pattern looks set to continue (Gibson, 1993; Kennedy, 1993; Yeomans et al., 1997). Some of these introduced parasites are known to be serious pathogens, which may have wide-ranging repercussions for conservation and fisheries management as well as for aquaculture (e.g. *Bothriocephalus acheilognathi* Yamaguti, 1934; see Williams, C.F., 2007. Impact assessment of non-native parasites in freshwater fisheries in England and Wales. Ph.D. thesis, University of Stirling, UK).

Of particular concern to fisheries is the ectoparasite *Gyrodactylus salaris* Malmberg, 1957 which can be highly pathogenic to Atlantic salmon, *Salmo salar* Linnaeus, 1758. This monogenean has caused a catastrophic decline of salmon stocks in Norway, decimating stocks in 46 rivers (see Table 2 of Bakke et al. (2007)) and leading to near extermination in five of these rivers (Mo, 1994). Gyrodactylid surveys conducted in the 1990s (Platten et al., 1994; Shinn et al., 1995) and on-going government-based surveillance programmes indicate that the UK is currently free of *G. salaris*. Nevertheless, there is considerable concern about the accidental introduction of this species, particularly since experimental exposure of native British salmon stocks to *G. salaris* in Norway demonstrated their susceptibility (Bakke and MacKenzie, 1993; MacKenzie and Bakke, 1994). Furthermore, *G. salaris* is now recorded from 13 neighbouring European countries, the most recent records originating from Poland (Rokicka et al., 2007) and Italy (Paladini et al., 2009b).

Comprehensive screening for *G. salaris* as a part of national monitoring programmes would generate huge numbers of samples, particularly in the event of a suspected outbreak. In order for monitoring to be effective, screening must rapidly identify specimens to the species level. However, *G. salaris* is notoriously difficult to discriminate from closely related and morphologically similar species present on European salmonids. If government policy seeks to maintain high standards of fish health and welfare in the UK, it is vital to have validated standard operating procedures (SOPs) for the efficient processing of specimens, while maintaining the highest possible likelihood of correctly identifying *G. salaris*. Several approaches have been used in the identification of *G. salaris*, besides the classical method of morphological examination of its haptor (attachment organ) under light microscopy. Morphometric analyses, which rely upon a range of statistical classifiers (Kay et al., 1999) and specific molecular techniques (Cunningham et al., 1995; Cunningham, 1997; Meinilä et al., 2002) have been developed to discriminate this pathogenic species from benign species also associated with salmonid hosts.

Morphometric discrimination of *Gyrodactylus* spp. can be difficult, due to the small size of taxonomically important structures (i.e. haptoral attachment hooks) and the importance of relatively small variations in diagnostic characters. Difficulty of identification is compounded by the extreme plasticity of these hooks and the potential for closely related species to hybridise (Bakke et al., 2007). Current estimates of identification performance have been made under artificially simplified conditions (Kay et al., 1999; McHugh et al., 2000; Shinn et al., 2000), with estimates based on controlled, small sets of specimens constructed from a limited number of populations analysed under ideal conditions. Standardised test sets used for determining classification efficiency normally preclude those specimens lost in preparation, difficult to measure, of poor quality for molecular analysis, or discarded because their identity was not confirmed. In addition, studies carried out to estimate classification efficiency for different techniques are often conducted in isolation using different individual specimens such that no direct comparisons can be made between methodologies. Under conditions of a suspected outbreak, numerous samples of *Gyrodactylus* requiring specific identification might be sent to a designated expert laboratory with a requirement for rapid and accurate identification.

The purpose of this study, therefore, was to conduct a double-blind trial to assess the classification performance of each of these morphometric and molecular methods, both singly and in combination, against a panel of gyrodactylid experts (i.e. visual identification) as techniques for the rapid identification of *G. salaris* and its accurate discrimination from other species of *Gyrodactylus* found on British salmonids, under conditions of a simulated outbreak. An improved understanding of the performance of these techniques under real-world conditions and of the parameters that affect their performance allows improvement of identification protocols and technologies and assists the development of robust SOPs. Such data can improve the monitoring and control of serious pathogens by British and Irish Fish Health Inspectorates and helps policy formulation, implementation and adherence.

2. Materials and methods

2.1. Origin of material

A total of 28 UK sites with salmonid populations were sampled for *Gyrodactylus* during routine electrofishing surveys and fish farm visits during February to May 2007. The following hosts were sampled: *Esox lucius* Linnaeus, 1758, *Oncorhynchus mykiss* (Walbaum, 1792), *S. salar*, *Salmo trutta fario* Linnaeus, 1758 and *Thymallus thymallus* (Linnaeus, 1758). Additional material included *Gyrodactylus* specimens collected from six salmonid populations in mainland Europe and from gyrodactylid populations maintained in research aquaria (Table 1). Where possible, entire fish ($n = 10$ per site; approximately 5 g in body weight) were taken and fixed in 96% research grade ethanol. For larger fish (i.e. >10 g in body weight), only the fins were removed and preserved. For each site, a random selection of gyrodactylids (usually 10–20) were picked off either the body or fins using triangular, mounted surgical needles and were placed in individually-labelled 1.5 ml Eppendorf tubes containing 96% ethanol. The time taken to screen each fish, and therefore the time taken to collect all of the specimens for the study, was recorded; the time taken to screen the small number of detached fin-only samples (Table 1), was not included in the time calculations. For each individual host fish, all *Gyrodactylus* present were removed and placed into two tubes, one for fins and one for those parasitising the body. In total, 620 gyrodactylids were harvested. Anonymised recoded tubes were then passed to another researcher. Variable numbers of *Gyrodactylus* were randomly taken from every tube. The randomly selected individuals were then prepared, as described in the following section, to provide 443 gyrodactylids for submission to the subsequent identification methodologies.

2.2. Sample preparation

Individual worms had their posterior attachment organ (i.e. haptor) excised using a scalpel under a dissecting microscope. Following excision, the body was transferred to a new 1.5 ml Eppendorf tube containing 96% ethanol while the haptor was subjected to proteolytic digestion to remove the tegument and musculature enclosing the haptoral armature. For the digestion step, the proteinase K-based method (Paladini et al., 2009a) (i.e. 100 μg/ml proteinase K (Cat. No. 4031-1, Clontech UK Ltd., Basingstoke, UK), 75 mM Tris–HCl, pH 8, 10 mM EDTA, 5% SDS) was used and the digestion of each gyrodactylid haptor was carefully monitored at 3× magnification on an Olympus SZ40 dissecting microscope. Once the tissues enclosing the haptoral hooks had been removed, digestion was arrested by the addition of 3 μl 50:50 formaldehyde:glycerine solution. A coverslip was added to the preparations ($n = 443$; 239 *Gyrodactylus derjavinoides* Malmberg, Collins, Cunningham & Jalali, 2007, 20 *Gyrodactylus lucii* Kulakovskaya, 1952, 41 *G. salaris*,