A comparison of *Saprolegnia parasitica* gene expression in the presence and absence of a host

by

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Abstract

As the importance of aquaculture increases globally, determining the infection strategies of opportunistic fish pathogens like *Saprolegnia parasitica* grows increasingly vital. *Saprolegnia parasitica*, a hemibiotrophic oomycete, has an initial biotrophic stage in which it suppresses host defences to facilitate infection, but the molecular mechanisms behind this infection strategy are largely unknown. In this study, I tested the hypothesis that expression of two *S. parasitica* virulence genes differs in the presence and absence of a host. The studied genes were: *SpHtp1,* a putative RXLR-like effector gene and *SPRG_13235*, a potentially horizontally transferred disintegrin gene with no known oomycete analogs. To test my hypothesis, I exposed pure *S. parasitica* zoospore cultures either to water imbued with *Salmo salar* effluents or to sterile water; then, I extracted RNA from experimental samples and measured expression differences with polymerase chain reactions (PCR). I successfully used nested PCR with gene-specific primers to qualitatively analyze expression, but quantitative analyses with real-time PCR (qPCR) were unsuccessful. Based on qualitative analyses, I concluded that *SPRG_13235* was expressed in low amounts in both host presence and host absence, implying that factors other than host presence mediate gene expression variance. I was unable to determine whether *SpHtp1* expression varied. Future studies should consider both studied genes but should focus on *SPRG_13235*, which remains largely uncharacterized. Disintegrin proteins inhibit vital cell functions by blocking integrin ligand-binding domains, potentially making them vital for host infection. The *SPRG_13235* primer sets designed in this study can be used to facilitate future research in this area and can also be used to effectively detect *S. parasitica* propagules in environmental samples.

Table of Contents

List of Tables

List of Figures

Acknowledgments

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Introduction

Host-pathogen relationships

Many oomycete and fungal pathogens are either necrotrophs or biotrophs (Jumpponen and Trappe, 1998; Thrall et al., 2007; Horbach et al., 2011). The ancestral pathogenic state is likely biotrophy, as most pathogens show features of biotrophy in some contexts (Spanu, 2012); nevertheless, this dichotomy allows classification. Necrotrophs, such as *Botrytis cinerea*, secrete enzymes and toxins that kill host cells, and they consume the resulting dead host tissue (Govrin and Levine, 2000; Glazebrook, 2005; Horbach et al., 2011). In contrast, biotrophs live within hosts and consume living tissues; thus, they do not immediately kill their hosts and have more subtle infection strategies (Glazebrook, 2005; Kliebenstein and Rowe, 2008; Horbach et al., 2011; Spanu, 2012). Biotrophs often penetrate living host tissue with specialized structures, such as appressoria, and may secrete specialized proteins to dampen host response (Kämper et al., 2006; Horbach et al., 2011). Recognizing these virulence factors is key to host defence against biotrophs (Kämper et al., 2006; Chisholm et al., 2006).

When attacked by a pathogen, hosts have numerous strategies. For example, plant hosts may kill localized areas of tissue (hypersensitive response) or may secrete proteases to degrade pathogenic proteins (Glazebrook, 2005; McGowan and Fitzpatrick, 2017). Hypersensitive responses generally work poorly against necrotrophs, which thrive on dead tissue (Glazebrook, 2005). Animal hosts, in contrast, often mount an initial inflammatory response, which is followed by a more specific adaptive immune response that contributes to future resistance (Chaplin, 2010).

Often, co-evolutionary arms races shape host-pathogen relationships (Altzier et al., 2003; Thrall et al., 2007), driving the evolution of matching gene families in pathogens and hosts

(Altzier et al., 2003; Glazebrook, 2005; Chisholm et al., 2006) and generating biodiversity (Case et al., 2005; Thrall et al., 2007). Nevertheless, pathogenicity exists on a complex gradient (Figure 1) (Jumpponen and Trappe, 1998; Thrall et al., 2007; Spanu, 2012), and different pathogen types employ vastly different strategies.

While obligate pathogens require a host for survival and persistence, facultative pathogens can exist as saprotrophs when hosts are absent, allowing them to persist in favourable environments until hosts become available. Thus, these pathogens are difficult to control and eradicate. Furthermore, these facultative pathogens are often generalists and can infect a wide range of potential hosts. Largely unaffected by the tight coevolution between a specialist pathogen and its host, many facultative generalist pathogens devastate weakened or stressed host populations (Noga, 1993). Stressful conditions can be abiotic or biotic in origin and include primary pathogenic infections, host reproductive periods, overcrowding, extreme temperatures, low moisture, pH changes, and pollution (Noga, 1993).

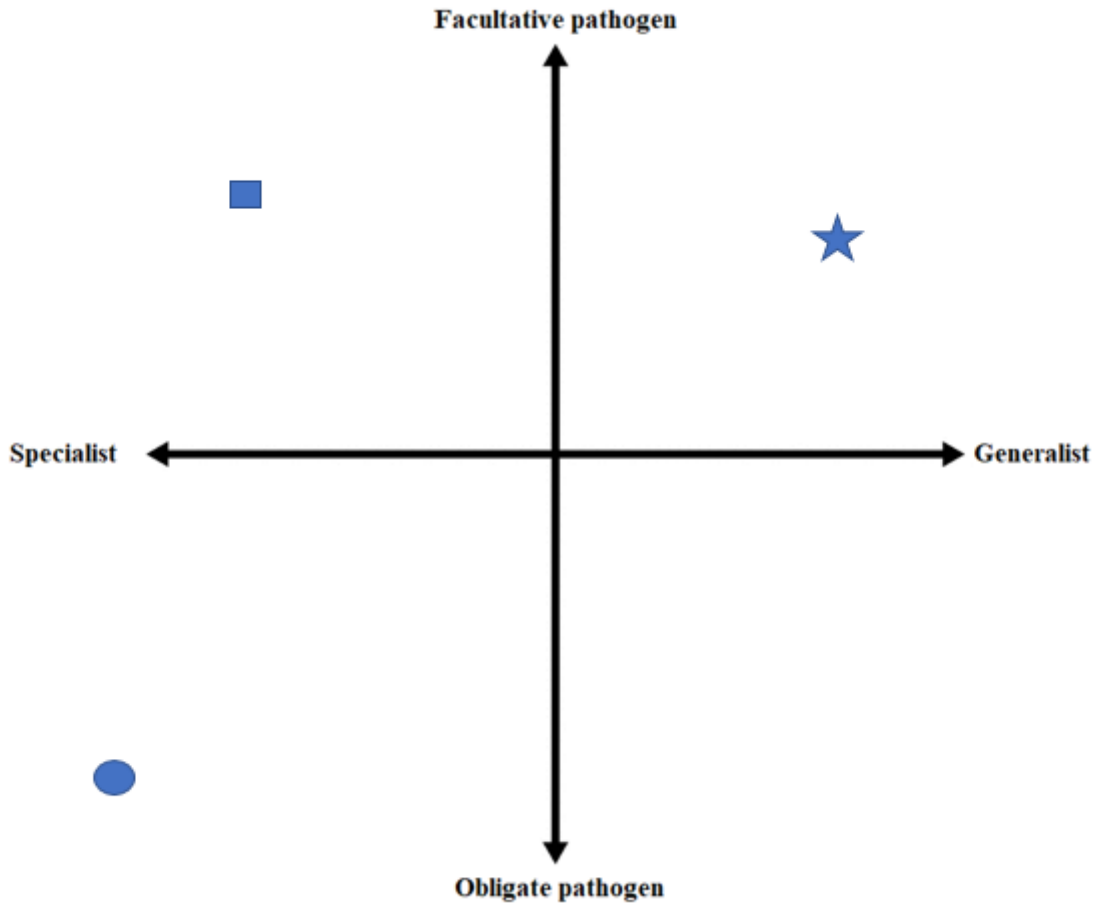

Figure 1: Simplified two-dimensional pathogenicity gradient for fungal and oomycete pathogens. Approximate location of *Saprolegnia parasitica*, the subject of this study, is marked with a star. For comparison, the obligately biotrophic oomycete *Hyaloperonospora arabidopsidis* (infects *Arabidopsis thaliana*) is marked with a circle, and the devastating oomycete plant pathogen *Phytophthora infestans* is marked with a square.

The question that my study explores is: how does gene expression in facultative pathogens change when a host is present? To answer this question, I evaluated how gene expression in the opportunistic oomycete animal pathogen *Saprolegnia parasitica* varied in the presence and absence of a host.

The Oomycota

Oomycetes, commonly known as "water moulds," are a group of saprotrophs, parasites, and pathogens (Judelson, 2012). Because they superficially resemble fungi physiologically and morphologically (Tyler, 2001; Walker and van West, 2007), oomycetes were originally taxonomically grouped with fungi (Baldauf et al., 2000; Beakes et al., 2012). However, the Oomycota belongs in the Heterokontophyta (Baldauf et al., 2000), a eukaryotic lineage that includes kelp and diatoms (Gunderson et al., 1987; Phillips et al., 2008). Modern heterokonts are defined primarily by their asexual zoospores, which have two flagella: a whiplash flagellum, which provides directionality, and a tinsel flagellum, which provides motility (Beakes, 1982; Walker and van West, 2007). All oomycetes retain these zoospores (Beakes, 1982), so they inhabit moist environments that permit zoospore release and transport.

Most oomycetes are saprotrophic and positively impact nutrient cycling in aquatic and moist soil ecosystems (Kamoun, 2003). However, many oomycetes are pathogenic. Characterized by rapid evolutionary rates, rapid development, and rapid dispersal, these pathogens are formidable (Walker and van West, 2007; Boutemy et al., 2011). The best studied oomycete pathogens are the economically important plant pathogens *Phytophthora* and *Pythium*, in the order Peronosporales and Pythiales (Kamoun, 2003; Yoshida et al., 2013). Nonetheless, species in the order Saprolegniales, especially *Saprolegnia* and *Aphanomyces* species, are emerging as economically important aquatic animal pathogens (van West, 2006; Phillips et al., 2008; Earle and Hintz, 2014).

The genus *Saprolegnia*

Saprolegnia is ubiquitous in aquatic environments worldwide but rarely inhabits terrestrial environments due to physiological constraints (Blaustein et al., 1994; Shearer et al., 2007). *Saprolegnia* species are generally difficult to delimit (Sandoval-Sierra et al., 2014). Previously, researchers used sexual characteristics to differentiate species, but *Saprolegnia* species do not always form sexual structures (Diéguez-Uribeondo et al., 2007; Sandoval-Sierra et al., 2014). More effective molecular identification techniques are currently used to differentiate *Saprolegnia* species (Diéguez-Uribeondo et al., 2007; Sandoval-Sierra et al., 2014; de la Bastide et al., 2015).

Saprolegniasis

Most *Saprolegnia* species are primarily saprotrophic (Yuasa and Hatai, 1995; Shearer et al., 2007), but some species opportunistically cause saprolegniasis (also called saprolegniosis), a geographically widespread disease. Most commonly, saprolegniasis affects adult salmonids (Pickering and Willoughby, 1982; Blaustein et al., 1994; van West, 2006; Phillips et al., 2008) and fish eggs (Thoen et al., 2011; Eissa et al., 2013; Songe et al., 2016). However, released hatchery-reared fish may spread saprolegniasis to amphibians (Kiesecker et al., 2001a, 2001b; Petrisko et al., 2008), and crayfish are also susceptible (Diéguez-Uribeondo et al., 1994; Krugner-Higby et al., 2010). Although several *Saprolegnia* species, such as *Saprolegnia ferax* (Kiesecker et al., 2001a, 2001b; Petrisko et al., 2008) and *Saprolegnia diclina* (Pickering and Willoughby, 1982), cause saprolegniasis, *Saprolegnia parasitica* is the most prevalent and virulent species on juvenile and adult fish hosts (Thoen et al., 2011; de la Bastide et al., 2015; Songe et al., 2016).

The *S. parasitica* life cycle involves both asexual and sexual stages (Figure 2). When nutrients are depleted, hyphal tips on the asexual mycelium form zoosporangia, which rapidly produce primary zoospores (Reid et al., 1995; van West, 2006; Phillips et al., 2008). These zoospores swim along chemical and electrical gradients, searching for potential hosts; when they detect a host, they encyst, rapidly losing their flagella and forming primary cell walls (Walker and van West, 2007). If this cyst attaches to a host, it germinates, penetrates the host, and forms a vegetative mycelium (Phillips et al., 2008). More often, the primary cyst does not attach to a suitable substrate and produces a secondary zoospore (Willoughby and Pickering, 1977; Reid et al., 1995). This phenomenon, called "polyplanetism," promotes survival and dispersal (Willoughby and Pickering, 1977; Reid et al., 1995; Daugherty et al., 1998; Phillips et al., 2008). The secondary zoospore is the most common host infection agent and forms a secondary cyst on its potential host (Phillips et al., 2008). Generally, bifurcated spines ornament this cyst, facilitating attachment and subsequent infection (Beakes, 1982; Hatai et al., 1990; Daugherty et al., 1998; Rezinciuc et al., 2018). When conditions are favourable, the secondary cyst germinates, forming a mycelium and starting disease development (Daugherty et al., 1998).

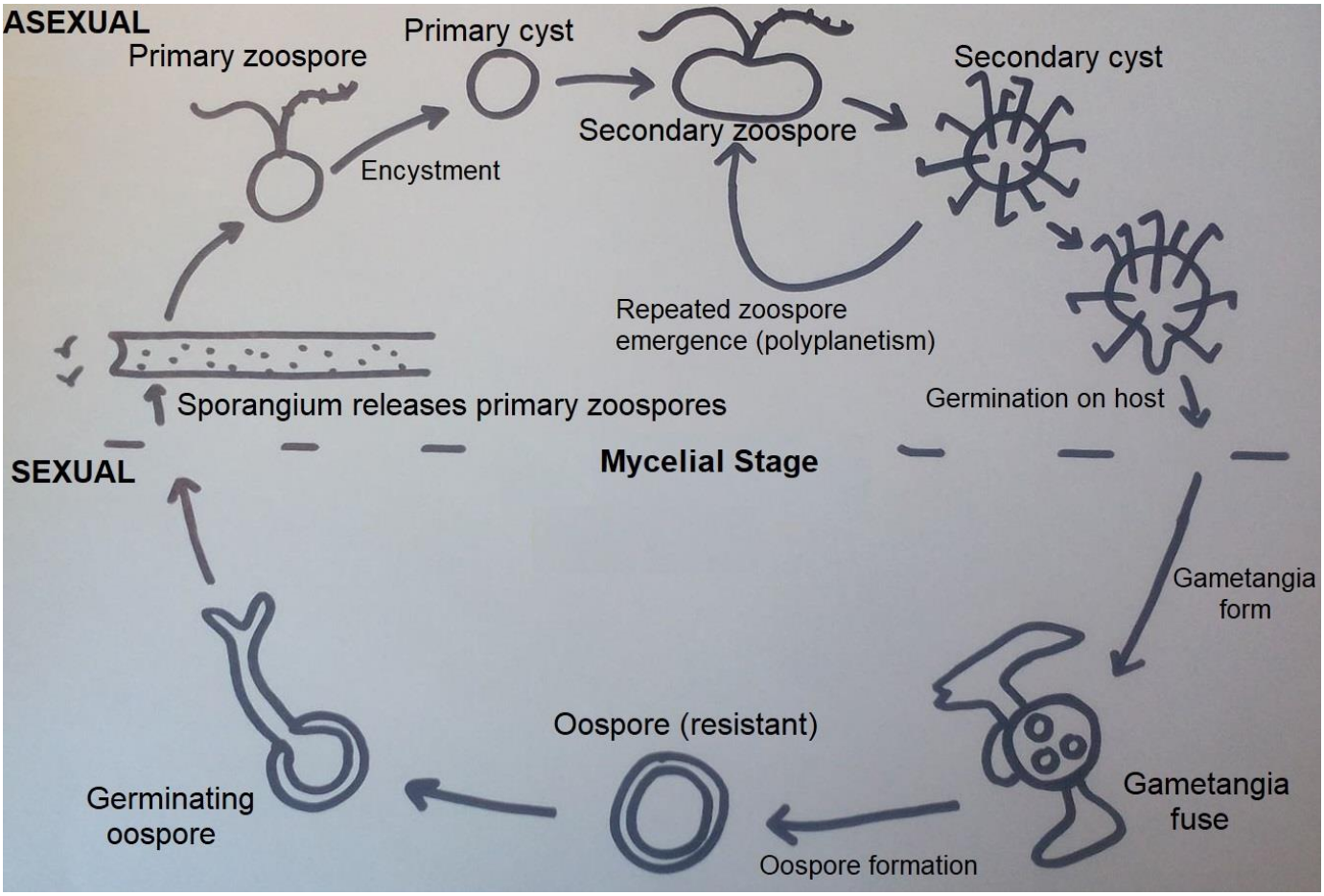

Figure 2: The generalized *Saprolegnia* lifecycle, adapted from van West (2006). The dotted line in the centre represents the mycelial stage.

Starting at the fish host's head or fins, this mycelium grows densely on the host's epidermis (Pickering and Willoughby, 1982; Hatai and Hoshiai, 1992; López-Dóriga and Martínez, 1998; van West, 2006). As the disease progresses, the epidermis degrades, allowing hyphae to enter the dermis, the hypodermis, the superficial musculature, and the blood vessels (Hatai and Hoshiai, 1992; López-Dóriga and Martínez, 1998; Hussein et al., 2002). This results in behavioural changes in the host, including erratic swimming and lethargy (Bruno and Stamps, 1987; Hatai and Hoshiai, 1992; Hussein et al., 2002). Eventually, because epidermal degradation changes fluid balance and ionic flow, severely impairing respiration (Bruno and Stamps, 1987),

the host dies of haemodilution (Torto-Alalibo et al., 2005; Roberge et al., 2007) and osmoregulatory failure (Pickering and Willoughby, 1982).

Overall, *S. parasitica* shows characteristics of both biotrophy and necrotrophy, making it a hemibiotroph. Often, *S. parasitica* infects its host without immediate damage and without eliciting a host immune response (Phillips et al., 2008); this indicates biotrophy. Currently, the exact mechanism for this stage is unknown. However, current evidence suggests that *S. parasitica* uses a host-targeting effector protein that is directly translocated into host tissues, where it modulates host response via an unknown mechanism (van West et al., 2010; Wawra et al., 2012a; Jiang et al., 2013). The subsequent epidermal degradation and host death indicates necrotrophy, which is likely achieved by secretion of proteases that degrade host tissues.

Saprolegnia parasitica most frequently causes saprolegniasis in stressed hosts. In natural environments, stresses include unfavourable temperatures (Snieszko, 1974; Bly et al., 1993), reproductive cycles and hormonal changes (Snieszko, 1974; Noga, 1993), major physiological changes (*e.g.* smoltification), intense competition and predation, primary pathogenic infections, and anthropogenic disturbances (Blaustein et al., 1994). In unnatural environments, such as aquaculture facilities, other stresses also contribute, making fish particularly susceptible to infection.

Importance in aquaculture

Saprolegnia parasitica is most destructive in aquaculture facilities for numerous reasons. First, overcrowding fish concentrates potential hosts, creating positive feedbacks. For example, Willoughby and Pickering (1977) found that zoospore concentration increased from 200 zoospores/L to over 22,000 zoospores/L when infected fish were added to experimental water

systems. Second, hatchery fish are more susceptible to physical damage such as abrasion (Bruno and Stamps, 1987), and wounds facilitate infections (Neish, 1977; Noga, 1993). Third, handling and vaccinations often stress fish (Noga, 1993). In extreme cases, *S. parasitica* becomes a primary pathogen, infecting both stressed and healthy fish by virtue of its abundance and persistence (Willoughby and Pickering, 1977; Hatai and Hoshiai, 1992).

Developing a safe control agent is, therefore, a current priority for the aquaculture industry. Malachite green, a highly effective control chemical, was banned because it is carcinogenic, and other control methods are less effective (van West, 2006; Phillips et al., 2008; Earle and Hintz, 2014). Although sodium chloride inhibits *S. parasitica* growth and is relatively harmless, the concentration required for its effectiveness is impractical (Khodabandeh and Abtahi, 2002). Other control chemicals, such as formalin, may have non-target effects if they leave the aquaculture facility (Schreier et al., 1996).

Molecular characterization of oomycetes

Oomycete host infection strategies

Plant pathogenic oomycetes are well-characterized molecularly and show a range of host infection strategies. *Pythium* species are usually necrotrophic and often cause necrosis in plant roots (McGowan and Fitzpatrick, 2017). *Phytophthora* species are often hemibiotrophic; therefore, they possess a wider range of virulence factors than necrotrophs and saprotrophs, which helps them evade host response during the initial biotrophic infection stage (McGowan and Fitzpatrick, 2017). Obligately biotrophic pathogens generally have fewer virulence factors than other oomycete pathogens, implying higher specificity (McGowan and Fitzpatrick, 2017).

Infection strategies: RXLR effector genes

Effectors are proteins secreted by a pathogen to modify host defences (McGowan and Fitzpatrick, 2017). These proteins degrade host cell walls or cell membranes, induce host cell necrosis, or modify host immune response (McGowan and Fitzpatrick, 2017). Two main types of effectors exist: apoplastic and cytoplasmic effectors (Schornack et al., 2009; Wawra et al., 2012b). Apoplastic factors act outside host cells, altering the external environment to encourage favourable infection conditions; for example, hydrolytic enzymes that break down host cell walls are apoplastic effectors (McGowan and Fitzpatrick, 2017). Cytoplasmic effectors act from within host cells (McGowan and Fitzpatrick, 2017). In the order Peronosporales, cytoplasmic effectors may be directly translocated into host cells via receptor-mediated endocytosis (Panstruga and Dodds, 2009; Schornack et al., 2009). These effectors then facilitate biotrophic infection by modifying host immune response from within host cells (Lamour et al., 2007; Whisson et al., 2007; Birch et al., 2008; Schornack et al., 2009; Grouffaud et al., 2010; Anderson et al., 2015). These vital proteins are secreted in Golgi-derived vesicles, and distinct N-terminal motifs often allow them to enter host cells (Panstruga and Dodds, 2009).

The most ubiquitous and essential effector gene family in the order Peronosporales is the RXLR family. Evolution and expansion of this gene family may have enabled biotrophy in a peronosporalean ancestor, leading to diversification (Anderson et al., 2015), but the origins of this family are ultimately unknown, and convergent evolution may have historically occurred. As plants adapt to oomycete pathogens, RXLR effector genes evolve in parallel, strongly suggesting that these genes are integral to host infection processes (Lamour et al., 2007; Schornack et al., 2009). RXLR effector proteins are characterized primarily by a conserved N-terminal RXLR motif (Whisson et al., 2007), which acts as a host translocation signal (Whisson et al., 2007;

Grouffaud et al., 2010). The C-terminal domains, however, are generally responsible for modifying host response and vary widely among species (Schornack et al., 2009). Currently, little is known about how RXLR effectors influence host response, as each pathogen produces unique proteins that have co-evolved alongside specific plant host populations (Panstruga and Dodds, 2009).

Molecular characterization of *Saprolegnia parasitica*

With an estimated genomic size of 63Mb and an estimated 17065 genes, *S. parasitica* genetically resembles other oomycetes (Jiang et al., 2013). However, its gene families are distinct, implying that pathogenic strategies differ in the order Peronosporales and the order Saprolegniales.

Notably, *S. parasitica* lacks the RXLR effector gene family (Jiang et al., 2013). Nevertheless, *S. parasitica* contains a gene (*SpHtp1*: "*Saprolegnia parasitica* host targeting protein 1") that codes for a host-targeting protein (SpHtp1) that resembles RXLR effectors and shares the distinctive N-terminal RXLR motif (van West et al., 2010; Wawra et al., 2012a; Jiang et al., 2013). This putative RXLR-like effector protein directly translocates into host cells and may dampen host immune response (van West et al., 2010; Wawra et al., 2012a; Jiang et al., 2013). *Saprolegnia parasitica* expresses *SpHtp1* during pre-infection stages; expression peaks soon after zoospores are exposed to host cells and decreases during encystment, implying that SpHtp1 mainly functions before infection (van West et al., 2010). To enter host cells, SpHtp1 interacts with tyrosine-O-sulfate cell-surface molecules rather than with phospholipids (Kale and Tyler, 2011; Wawra et al., 2012a). Currently, the functions of RXLR effectors have only been characterized for plant pathogenic oomycetes.

The *S. parasitica* genome also contains potential virulence genes without known analogs in other oomycetes. Interestingly, *S. parasitica* has several animal-like gene families, such as a galactose-binding lectin family, a disintegrin family, and a haemolysin E family, that potentially arose through horizontal gene transfer (HGT) from bacteria (Jiang et al., 2013; Srivastava et al., 2018).

Disintegrins, low molecular weight and cysteine-rich proteins, may facilitate pathogenic infection processes (Paine et al., 1992; Barja-Fidalgo et al., 2005; Hammouda et al., 2016). Disintegrins have diverse functions, but all are competitive inhibitors of integrin ligand-binding domains (Barja-Fidalgo et al., 2005). Because functioning integrins often integrate cytoskeletal elements with the extracellular matrix, they are vital for signal transduction in animal cells; thus, disintegrin-mediated inactivation of integrins may drastically impact cell functions (Barja-Fidalgo et al., 2005; Gálan et al., 2008; Selistre-de-Araujo et al., 2010). Furthermore, integrins are vital in many immune system functions, such as diapedesis (Tanaka et al., 1993; Barja-Fidalgo et al., 2005).

Nothing is currently known about how disintegrins mediate host-pathogen relationships in oomycete pathogens. However, previous work on disintegrins isolated from viper and pit viper (Family Viperidae) venoms suggests that disintegrins may profoundly influence immune response (Barja-Fidalgo et al., 2005). First, disintegrins may prevent immune cell migration, which is largely mediated by integrins (Tanaka et al., 1993). Second, disintegrins may suppress adaptive immune responses. Integrins modulate helper T-lymphocyte (T-cell) co-activation by rapidly accommodating T-cell attachment or detachment (Hogg et al., 2003); disintegrin-mediated inactivation of integrins can, therefore, prevent T-cell functions (Barja-Fidalgo et al., 2005). Fish infected by *S. parasitica* often show a dampened adaptive immune response, even if

they mount a strong inflammatory response (Belmonte et al., 2014); possibly, disintegrins help *S. parasitica* achieve this.

Study rationale and hypotheses

Saprolegnia parasitica genetics studies usually only examine differential gene expression at different life stages. Evaluating gene expression in host presence and absence at a given life stage may be equally valuable because *S. parasitica* is opportunistically pathogenic and may express different genes when acting as a saprotroph. Due to its flexible lifestyle, *S. parasitica* may transition between life stages more rapidly when exposed to potential hosts (Noga, 1993). In this study, I will evaluate expression of two potential *S. parasitica* virulence genes in the presence and absence of a host.

First, I will evaluate *SpHtp1* expression in host presence and absence. Other oomycete pathogens express RXLR effector genes during biotrophic infection stages (Schornack et al., 2009). *SpHtp1* follows similar patterns to other oomycete cytoplasmic effectors despite its apparent lack of homology, implying that it is essential for host infection. Thus, I hypothesize that *SpHtp1* will be expressed more in a host's presence.

Second, I will evaluate disintegrin gene expression. Because disintegrins potentially interact with integrins to modulate host immune response, they may also facilitate biotrophic infection. Thus, I hypothesize that disintegrin genes will be more highly expressed in a host's presence. *Saprolegnia parasitica* has 16 disintegrin genes; I evaluated the gene *SPRG_13235* because it is highly expressed in infection stages (Jiang et al., 2013).

Evaluating these genes may reveal that *S. parasitica* expresses different genes when in an environment conducive to a saprotrophic lifestyle (host absence) than when in an environment conducive to a pathogenic lifestyle (host presence).

Methodology

Inoculum preparation

To obtain pure oomycete cultures, *Saprolegnia parasitica* was isolated from aquaculture water samples. In a biosafety cabinet, 5 mL of water from an aquaculture facility was added to sterile hemp seeds in a sterile Petri dish. Petri dishes were incubated at room temperature (21˚C) until oomycete growth on hemp seeds was apparent. Colonized seeds were plated on glucose-peptone (GP) agar medium. Antibiotic (Rifampicin [50µg/mL], Streptomycin [10µg/mL], and Chloramphenicol [25µg/mL]) and antifungal (Nystatin [10µg/mL]) compounds were added to the GP to discourage contaminant growth. Culture identities were confirmed via internal transcribed spacer (ITS) barcoding on subsequently extracted DNA.

To prepare liquid cultures, two ~5mm diameter plugs of *S. parasitica* were aseptically transferred from the GP plates to 500mL of sterile glucose-yeast extract (GY) liquid medium in 1L flasks. These flasks were incubated for three days at room temperature on an Orbit Shaker (LabLine) set at 150 rotations per minute. Then, zoospore release was induced. The GY medium was poured off, leaving only prominent mycelial balls, and 250mL of 0.5mM $CaCl_2$ was added to each culture. The cultures were then incubated for 2 hours on the Orbit Shaker in a 15˚C refrigerator. The $CaCl_2$ was then poured off and replaced with fresh solution, and cultures were incubated for 2 more hours in a 15˚C refrigerator. After this incubation, the $CaCl_2$ solution was replaced again, and the flasks were incubated at room temperature on the Orbit Shaker for 2-10 days.

Zoospore concentration was checked daily using standard cell counting techniques. (10^6 cells per litre was the desired minimum concentration for experimental use.) After liquid cultures were thoroughly mixed, 5 mL samples were taken from each culture, and 10µL sub-samples

were examined with a haemocytometer. For each sample, the number of cells in five haemocytometer secondary squares was counted. Zoospore concentration (cells/L) in each culture was determined using the following formula:

$$N = \{(\text{cells counted in 5 cells}) / [(0.02\text{mm}^3) \text{ X } (1000\text{mL/mm}^3)]\} \text{ X } 1000\text{mL/L}$$

Experimental design and nucleic acid extractions

Experiments followed a paired design with two treatments and four independent replicates (8 samples total). Each of four cultures was subjected to two different treatments, and data were analyzed taking similarities between dependent samples into account. The two treatments were host presence (HPT) and host absence (HAT). For the HPT, 75mL of liquid *S. parasitica* culture was added to 75mL of water from a water system in the University of Victoria's outdoor aquatics facility that contains *Salmo salar* individuals. For the HAT, 75mL of the same culture was added to 75mL of clean water from the same facility. All experiments were performed at 15˚C one day after zoospore formation. Each culture was named according to the date of zoospore induction (November 18: 1118) and the treatment used (HPT or HAT); an arbitrary culture identification number (1-4) distinguished independent replicates. For example, paired samples from culture 1 were named 1118-1 (HPT) and 1118-1 (HAT), while samples from culture 2 were named 1118-2 (HPT) and 1118-2 (HAT).

After samples were mixed, they incubated for one hour. Then, RNA was extracted from each sample using the RNeasy mini kit (Qiagen); because zoospores are small and delicate, a protocol for yeast-like cells was used. To concentrate *S. parasitica* cells, each sample was centrifuged in 50mL increments at 1000 x *g* for 5 minutes. After each centrifugation, the top 45mL of water was removed and replaced with fresh sample. Concentrated cells were

mechanically disrupted in a Mini Beadbeater (BioSpec) for 1 minute before RNA extraction protocols were followed. After extraction, a Nanodrop was used to test RNA purity and concentration. Extracted RNA was stored in a -20˚C freezer.

To prepare genomic DNA controls for polymerase chain reactions (PCR), DNA was extracted from pure *S. parasitica* cultures using PrepMan® Ultra Sample Preparation Reagent (ThermoFisher Scientific). First, 15mL of pure liquid culture was centrifuged for 2 minutes at 14000 rpm to concentrate zoospores. Then, the top 14 mL of liquid was removed, and 1mL of PrepMan solution was added to the sample. This sample was thoroughly vortexed and was heated for 10 minutes on a 100˚C heating block. Then, the samples were cooled for 2 minutes before being centrifuged for 2 minutes at 14000 rpm. Extracted DNA was diluted 10X with sterile distilled water.

Polymerase chain reactions (PCR) protocols

Reverse transcription to form complementary DNA (cDNA)

To convert RNA samples to complementary DNA (cDNA), the 5X All-In-One Reverse Transcription Mix (ABM) was used. 4µL 5X and 10µL RNase free water were added to 6µL of each RNA sample. Then, samples were incubated for 10 minutes at 25˚C and 50 minutes at 42˚C. Reactions were stopped by placing samples on an 85˚C heating block for 5 minutes. cDNA samples were cooled briefly on ice before they were stored in a -20˚C freezer.

Primer design

Primers were designed both for the two target genes (*SPRG_13235* and *SpHtp1*) and for a housekeeping gene. *SpTub-b*, which codes for the β-tubulin gene in *S. parasitica* was chosen as a

housekeeping gene because it is consistently expressed at all life stages (van West et al., 2010). This gene was used to assess cDNA quality and to standardize gene expression levels in the real-time PCR (qPCR) analysis.

Primer sets were designed using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). All primer sets had a guanine/cytosine (G/C) content of 50-60% and a melting temperature of 60-70˚C. All primers were suitable for amplifying both genomic DNA and cDNA. Primer specificity was assessed using SnapGene.

Nested protocols

Nested protocols were used to facilitate detection of low gene expression levels from low concentration cDNA templates. Therefore, two gene-specific primer sets were used for each target gene (*SPRG_13235, SpHtp1,* and *SpTub-b*). The first set amplified a relatively long DNA sequence (Table 1). These amplicons will hereafter be referred to as “step-one amplicons.” Step-one amplicons were then used as DNA templates for a PCR protocol that used a second primer set. The second primer set amplified a shorter region “nested” within the sequence amplified by step-one primers (Table 2). These amplicons will hereafter be referred to as “step-two amplicons.” The nesting protocols used for *SPRG_13235, SpHtp1,* and *SpTub* are shown in Figures 3, 4, and 5 respectively.

Table 1: Characteristics of step-one PCR primer sets used to amplify target DNA sequences.

Gene amplified	Primer sequences (5'→3')	Target length	Melting temperature (T_M)
SPRG_13235	TGAAGCTGCTCTGGGTTTGC (Forward) CCAAAGGTACTGCACGAGGT (Reverse)	181bp	62.4˚C
SpHtp1	CCACCTTCGGAGTGATAGCC (Forward) GAATGTCGGGACGACCTGGG (Reverse)	454bp	60.4˚C (Forward) 58.4˚C (Reverse)
SpTub-b	CGAGTGGATCCCGAACAACA (Forward) TGGTACTGCTGGTACTCCGA (Reverse)	252bp	62.4˚C

Table 2: Characteristics of step-two PCR primer sets used to amplify target DNA sequences.

Gene amplified	Primer sequences (5'→3')	Target length	Melting temperature (T_M)
SPRG_13235	GGCATGGACCTTCTGGCA (Forward) TACTGTGGCACGTCAGTTGC (Reverse)	112bp	62.2 ˚C (Forward) 62.4˚C (Reverse)
SpHtp1[1]	CGTCATCATCGGAGAAATCC (Forward) CGCTTTGTTCAAGTTGTTCC (Reverse)	104bp	60.4˚C (Forward) 58.4˚C (Reverse)
SpTub-b[1]	AGGAGATGTTCAAGCGCGTC (Forward) GATCGTTCATGTTGGACTCGGC (Reverse)	129bp	62.4˚C (Forward) 64.5˚C (Reverse)

[1]Designed by van West et al. (2010)

TGAAGCTGCTCTGGGTTTGCGTCGCGGCCAACGCGATCGGGCTCTCGCTCGGCAT
GGACCTTCTGGCATCCGAAACGGCTCCGGTCGGCGCGAATCTCTTGGCGGCCGCG
GCCACGAAGTGCACGCGTGACAACGACTGCCCGCAACTGACGTGCCACAGT**ACCT**
CGTGCAGTACCTTTGG

Figure 3: DNA sequence amplified by *SPRG_13235* nesting protocol. Step-one primers are in bold, and step-two primers are underlined.

CCACCTTCGGAGTGATAGCCGCGGCCACCGCCACCACGCCCATCATGG
ACAAGTTATCGACAAGGAAAACAACAACTCGCAGGAGCAAGCCACGAC
TGGCAACAGCGTCGAGACCAACCAAGTGCCATCCACCGAGCCGACGAA
AGACAAGACAACTCCGATGAAGAACGCTTTGTTCAAGTTGTTCCGCGA
AAAGAAACTCAAAACCAAAAATGCTGGCAACGGGCATGCTCACGACGA
CGACGACGATTCGGATTTCTCCGATGATGACGTACCGACAAATGCTCC
CACAGACGCTCCCACGGGCGCGCCTACTGATGCTCCGACCGATGCTCC
GACCGTAGCACCCACCGACGCTCCTACCGACGCTCCCACCGAAGCACC
TACCAACGCGCCTACCGGTACCGATGCCCCGACCGATGCTCCCACGGA
CG**CCCAGGTCGTCCCGACATTC**

Figure 4: DNA sequence amplified by *SpHtp1* nesting protocol. Step-one primers are in bold, and step-two primers are underlined.

CGAGTGGATCCCGAACAACATCAAGGCCAGCGTGTGCGACATCCCGCCGAAGGGC
CTTAAGATGTCGACGACGTTCATCGGTAACTCGACGGCGATCCAGGAGATGTTCA
AGCGCGTCTCGGAGCAGTTCACGGCCATGTTCCGGCGGAAGGCTTTCTTGCATTG
GTACACGGGCGAAGGCATGGACGAGATGGAGTTCACGGAAGCCGAGTCCAACATG
AACGATCTCGTC**TCGGAGTACCAGCAGTACCA**

Figure 5: DNA sequence amplified by *SpTub-b* nesting protocol. Step-one primers are in bold, and step-two primers are underlined.

PCR protocols

Transcription was assessed qualitatively using standard PCR with individual cDNA samples. All reactions occurred in an Eppendorf Mastercycler® Gradient model 5331. DreamTaq reagents (ThermoFisher) were used in all reactions, and 2.0µL of template DNA was used in each 10µL reaction (negative controls received distilled water instead of DNA template). Final primer concentrations were 0.5µM, and final dNTP concentration was 0.2µM. Amplicons were run via gel electrophoresis for 45-60 minutes at 80 volts on a 2.0-3.0% agarose gel in 1X

Tris-Acetate-EDTA (TAE) buffer. Gels were stained using GelRed, and were visualized with Gel Doc XR+ and ImageLab.

Annealing temperatures were determined by performing PCR with annealing temperature gradients; the annealing temperatures that showed consistent amplification of the correct products and no non-specific binding were used in subsequent reactions. General PCR cycling conditions were determined using genomic DNA template (Table 3), and conditions were optimized with pooled cDNA template (Table 4). Pooled cDNA consisted of a mixture of all experimental cDNA samples. Then, each cDNA sample was individually evaluated with each primer set. At least one amplicon from each nesting protocol was purified with a Column-Pure PCR Clean-up Kit (ABM) and sent it to Eurofins Genomics to be sequenced. Basic Local Alignment Search Tool (BLAST) analysis was used to ensure that the sequenced amplicons were the predicted products.

Table 3: PCR conditions used for genomic DNA templates.

Primer set	**Cycle conditions**
Step 1: *SpHtp1*	N/A
Step 1: *SPRG_13235*	Initial denaturation: 95˚C for 2 min 45 CYCLES OF: -30s at 95˚C -30s at 58.6˚C -12s at 72˚C Final extension: 72˚C for 2 min
Step 1: *SpTub-b*	Initial denaturation: 95˚C for 2 min 40 CYCLES OF: -30s at 95˚C -30s at 55.8˚C -16s at 72˚C Final extension: 72˚C for 2 min
Step 2: *SpHtp1*	N/A
Step 2: *SPRG_13235*	Initial denaturation: 95˚C for 2 min 40 CYCLES OF: -30s at 95˚C -30s at 54.8˚C -8s at 72˚C Final extension: 72˚C for 2 min
Step 2: *SpTub-b*	Initial denaturation: 95˚C for 2 min 40 CYCLES OF: -30s at 95˚C -15s at 55.8˚C -8s at 72˚C Final extension: 72˚C for 2 min

NOTE: *SpHtp1* primers did not effectively amplify genomic DNA templates.

Table 4: PCR conditions used for cDNA templates.

Primer set	**Cycle conditions**
Step 1: *SpHtp1*	N/A (unsuccessful)
Step 1: *SPRG_13235*	Initial denaturation: 95˚C for 2 min 45 CYCLES OF: -30s at 95˚C -30s at 58.6˚C -12s at 72˚C Final extension: 72˚C for 2 min
Step 1: *SpTub-b*	Initial denaturation: 95˚C for 2 min 40 CYCLES OF: -30s at 95˚C -15s at 55.8˚C -16s at 72˚C Final extension: 72˚C for 2 min
Step 2: *SpHtp1*	Initial denaturation: 95˚C for 2 min 45 CYCLES OF: -30s at 95˚C -30s at 54˚C -7s at 72˚C Final extension: 72˚C for 2 min
Step 2: *SPRG_13235*	Initial denaturation: 95˚C for 2 min 45 CYCLES OF: -30s at 95˚C -30s at 54.8˚C -8s at 72˚C Final extension: 72˚C for 2 min
Step 2: *SpTub-b*	Initial denaturation: 95˚C for 2 min 40 CYCLES OF: -30s at 95˚C -15s at 55.8˚C -8s at 72˚C Final extension: 72˚C for 2 min

qPCR protocols

The standard-curve qPCR method was used to analyze relative transcription of the desired genes compared to a housekeeping gene (*SpTub-b*) based on cycle threshold (Ct) values. First, a dilution series (1, 1/2, 1/4, 1/8, 1/10) was prepared from either pooled cDNA or standard PCR step-one amplicons. Second, these serial dilutions were run in triplicate using the conditions described in Table 4. Each primer set was individually tested, and standard curves were created for each primer set. Then, individual cDNA samples were run in triplicate, and gene expression was evaluated using constructed standard curves.

All reactions took place in a StepOnePlus RT-PCR System (ThermoFisher). Reactions were performed using PowerUp SYBR Green Master Mix (ThermoFisher) reagents, and 1.5µL of DNA template was used in each 10µL reaction (negative control samples received distilled water instead of cDNA template). The same primers used for standard PCR were used in qPCR, but variable primer concentrations were used.

Data analysis

qPCR analysis

Quantitative data were analyzed using the analytical methods described by Livak and Schmittgen (2001). For each cDNA sample, Ct values of both the desired gene (*SPRG_13235* or *SpHtp1*) (DCt) and the *SpTub-b* gene (HCt) were acquired from the constructed standard curves. For each treatment, ΔCt (DCt – HCt = ΔCt) was calculated, and differences between HPT and HAT treatments for each culture were assessed using ΔΔCt (experimental (HPT) ΔCt value (ΔCtE) – control (HAT) ΔCt value (ΔCtC)). Then, the formula $\mathbf{2^{-\Delta\Delta Ct}}$ was used to estimate

"expression-fold change" between treatments. Mean $2^{-\Delta\Delta Ct}$ values from all experimental replicates were used to estimate true transcription differences between treatments.

Results

RNA concentration and purity

RNA concentration and purity varied (Table 5). Concentrations ranged from 2.0ng/µL to 23.5ng/µL, and purity (260/280) varied from pure (*e.g.* 2.15) to impure (*e.g.* 1.50). However, subsequent nesting protocols aimed to normalize cDNA quality.

Table 5: Concentration and purity of extracted RNA. Dependent (paired) samples share a culture ID number.

Culture ID	Treatment	Zoospore count (cells/L)	RNA concentration (ng/µL)	260/280
1118-1	HPT (FW)	8.8 X 10^10	6.8	1.76
1118-2	HPT (FW)	1.1 X 10 ^11	10.8	1.50
1118-3	HPT (FW)	4.0 X 10^10	23.5	1.47
1118-4	HPT (FW)	1.7 X 10^9	17.3	1.62
1118-1	HAT (TW)	8.8 X 10^10	4.8	1.61
1118-2	HAT (TW)	1.1 X 10^11	2.9	2.33
1118-3	HAT (TW)	4.0 X 10^10	5.3	2.15
1118-4	HAT (TW)	1.7 X 10^9	22.1	1.51

Primer optimization

SPRG_13235 primers

The designed *SPRG_13235* step-one primers worked effectively on *S. parasitica* genomic DNA templates. Non-specific binding (producing a 300bp product) occurred at lower annealing temperatures but decreased at higher annealing temperatures (Figure 6). A 30s annealing time was optimal; decreasing annealing time did not further reduce non-specific binding.

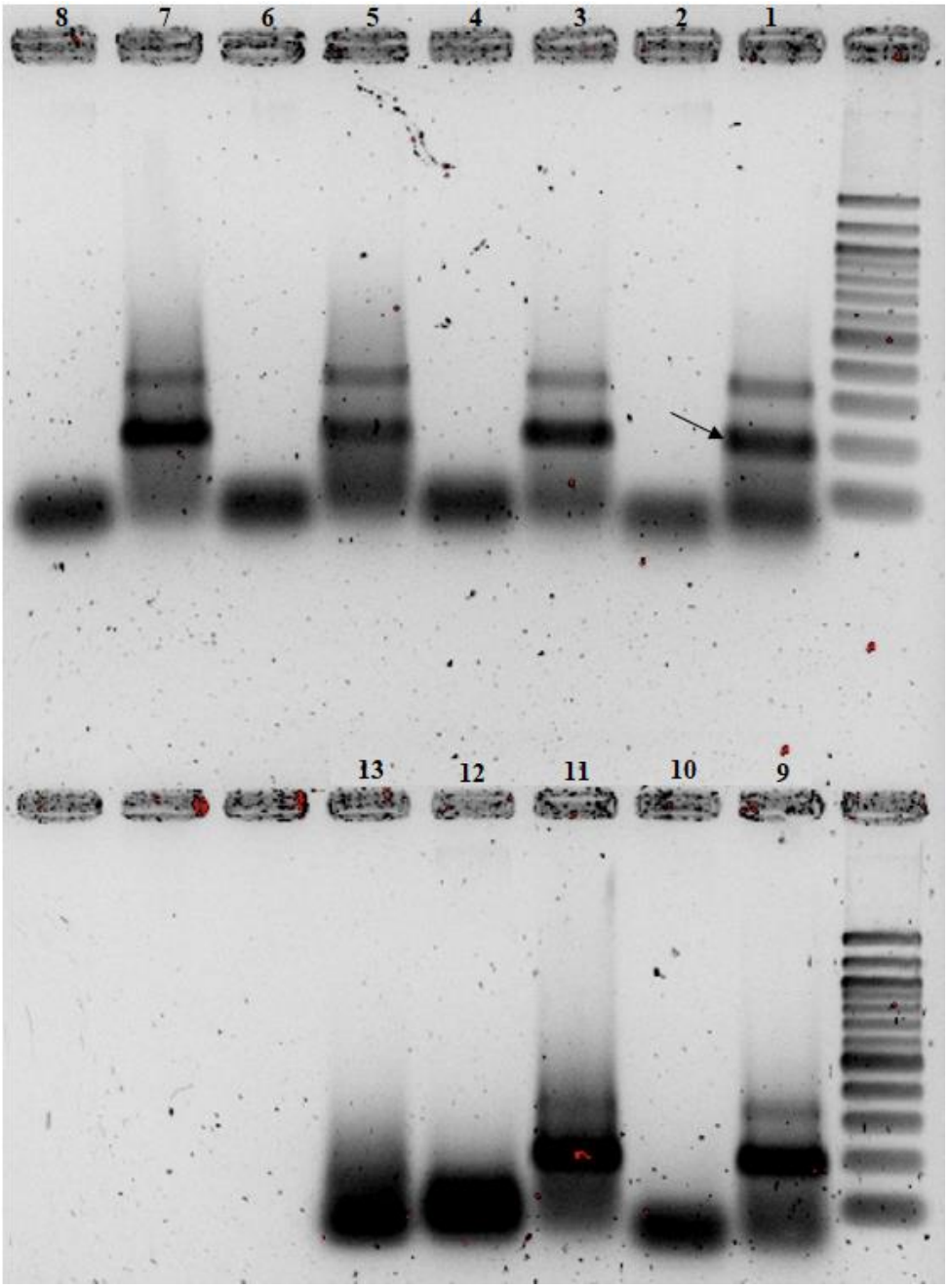

Figure 6: Step-one amplicons of the *SPRG_13235* nesting protocol. This image shows an annealing temperature gradient with alternating genomic and pooled cDNA samples (1=genomic DNA 48.4˚C; 2=cDNA 48.4˚C; 3=genomic DNA 50.6˚C; 4=cDNA 50.6˚C; 5=genomic DNA 52.8˚C; 6=cDNA 52.8˚C; 7=genomic DNA 54.8˚C; 8=cDNA 54.8˚C; 9=genomic DNA 56.5˚C; 10=cDNA 56.5˚C; 11=genomic DNA 58.6˚C; 12=cDNA 58.6˚C; 13=negative control at 48.4˚C). The non-specific ~300bp product decreases in intensity with increasing annealing temperature, while the desired product (~200bp: marked with an arrow in well 1) intensifies. A 100bp ladder is shown on the right of each row.

As seen in Figure 6, pooled cDNA template did not show amplification of *SPRG_13235* with step-one primers, necessitating a nesting protocol. The nesting protocol was highly effective; step-one amplicons were an effective template for step-two primers, and step-one amplicons from both cDNA and genomic DNA amplified well (Figure 7). I did not observe non-specific binding.

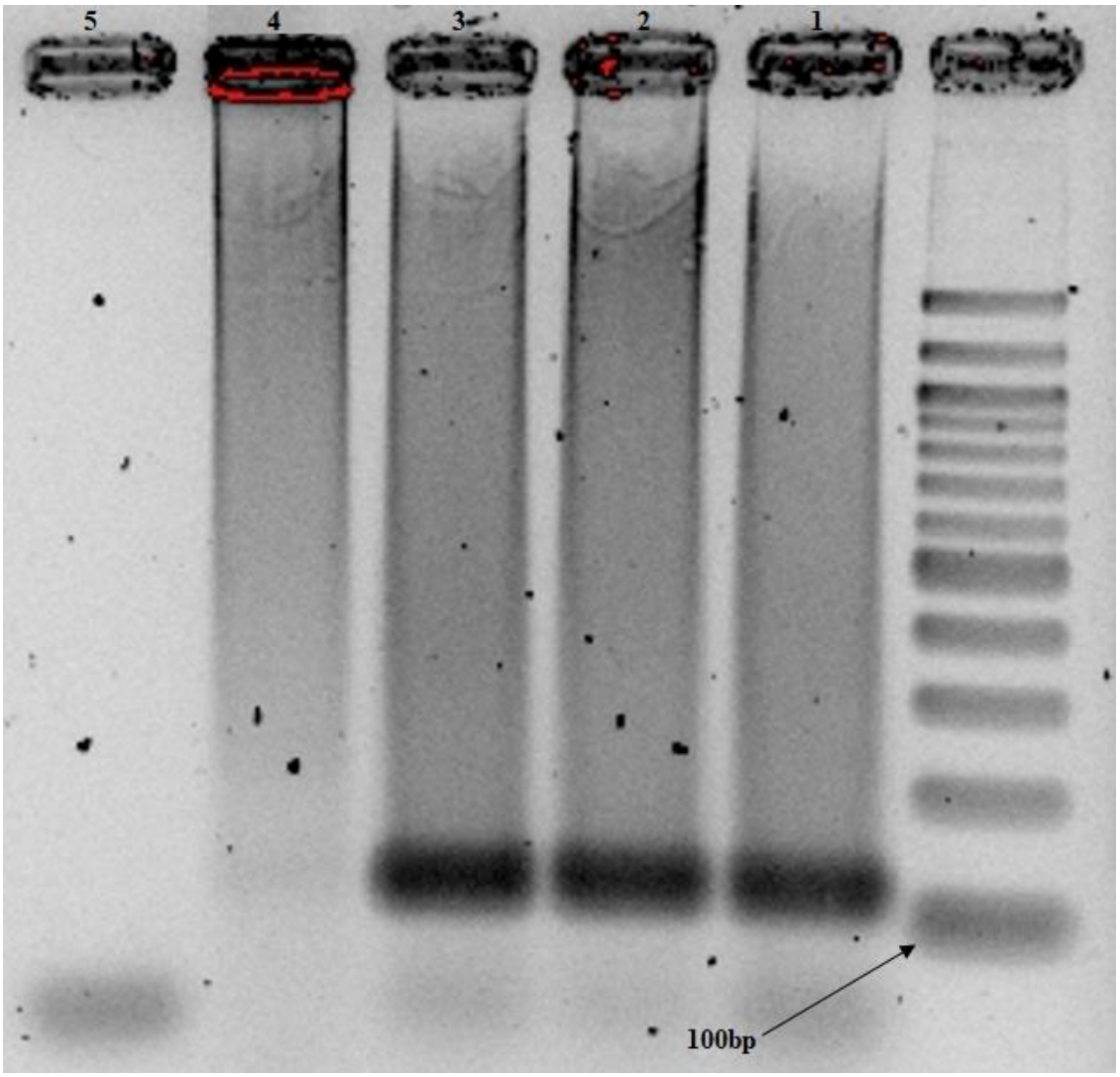

Figure 7: Step-two amplicons of the *SPRG_13235* nesting protocol on an annealing temperature gradient (1=genomic DNA step-one amplicon template 54.8˚C; 2=cDNA step-one amplicon template 54.8˚C; 3=genomic DNA step-one amplicon template 56.5˚C; 4=cDNA step-one amplicon template 56.5˚C; 5=negative control). A 100bp ladder is shown on the far right.

SpHtp1 primers

Although *SpHtp1* primers were designed to amplify both cDNA and genomic DNA templates (see van West et al., 2010), they did not effectively amplify genomic DNA templates in any tested conditions. Thus, I only tested *SpHtp1* primers with pooled cDNA.

The step-one primers were highly non-specific, and many anomalous bands (including potential primer dimer bands) appeared when PCR amplicons were visualized on agarose gels (Figure 8). Thus, I only used step-two primers to amplify *SpHtp1* in cDNA samples and was unable to use the designed nesting protocol. Step-two primers were more specific and did not produce any visible non-specific binding when tested with pooled cDNA samples at an annealing temperature of 54˚C (Figure 9).

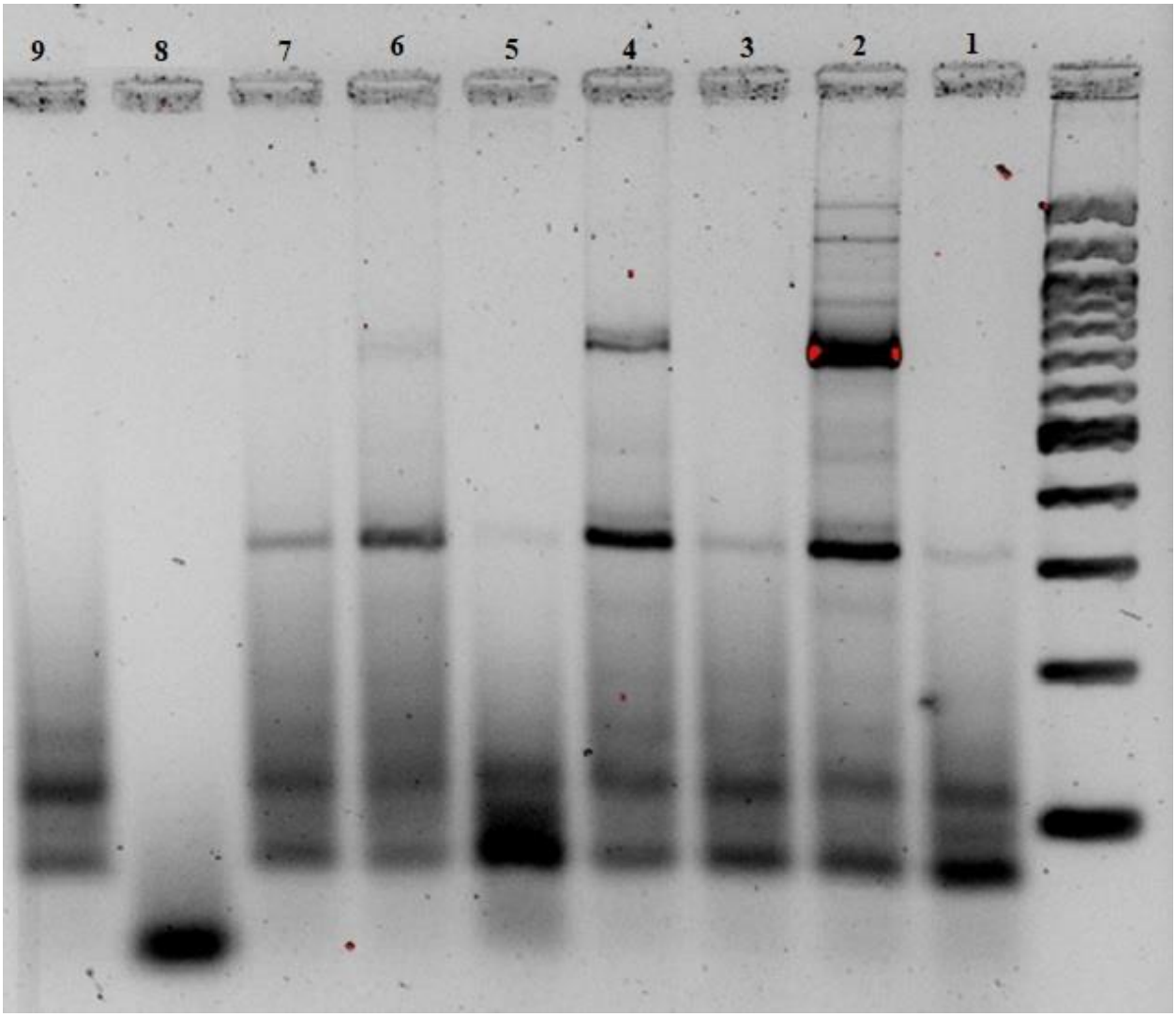

Figure 8: Highly variable amplicons produced by *SpHtp1* step-one primers. Amplicons are pooled cDNA samples tested with different annealing temperatures (all other cycling parameters constant). (1=48.4˚C; 2=50.6˚C; 3=52.8˚C; 4=54.8˚C; 5=56.5˚C; 6/7=58.6˚C (shown twice due to pipetting error); 8=negative control 48.4˚C; 9=genomic DNA 48.4˚C). A 100bp ladder is shown on the far right.

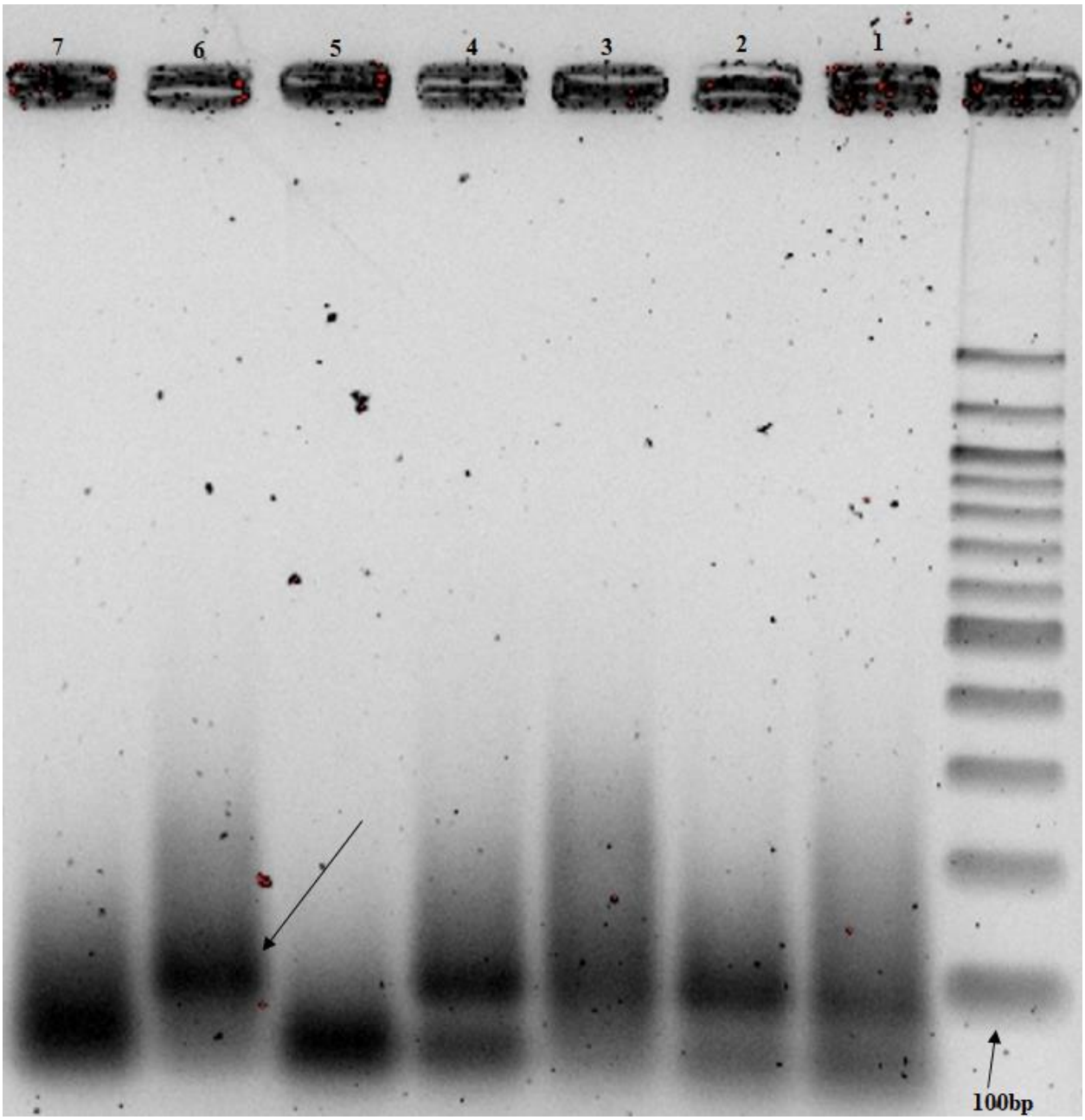

Figure 9: *SpHtp1* amplification observed when step-two primers were used in isolation on an annealing temperature gradient (1=genomic DNA 50˚C; 2=cDNA 50˚C; 3=negative control 50˚C; 4=cDNA 52˚C; 5=negative control 52˚C; 6=cDNA 54˚C; 7=negative control 54˚C). Arrow indicates the desired band size (~104bp). A 100bp ladder is shown on the far right.

SpTub-b primers

Step-one *SpTub-b* primers specifically amplified genomic DNA when many different annealing temperatures were tested (Figure 10). However, when identical conditions were tested with cDNA templates, amplification was more variable (Figure 11). Thus, I used different PCR conditions for cDNA, which eliminated non-specific binding (Figure 12).

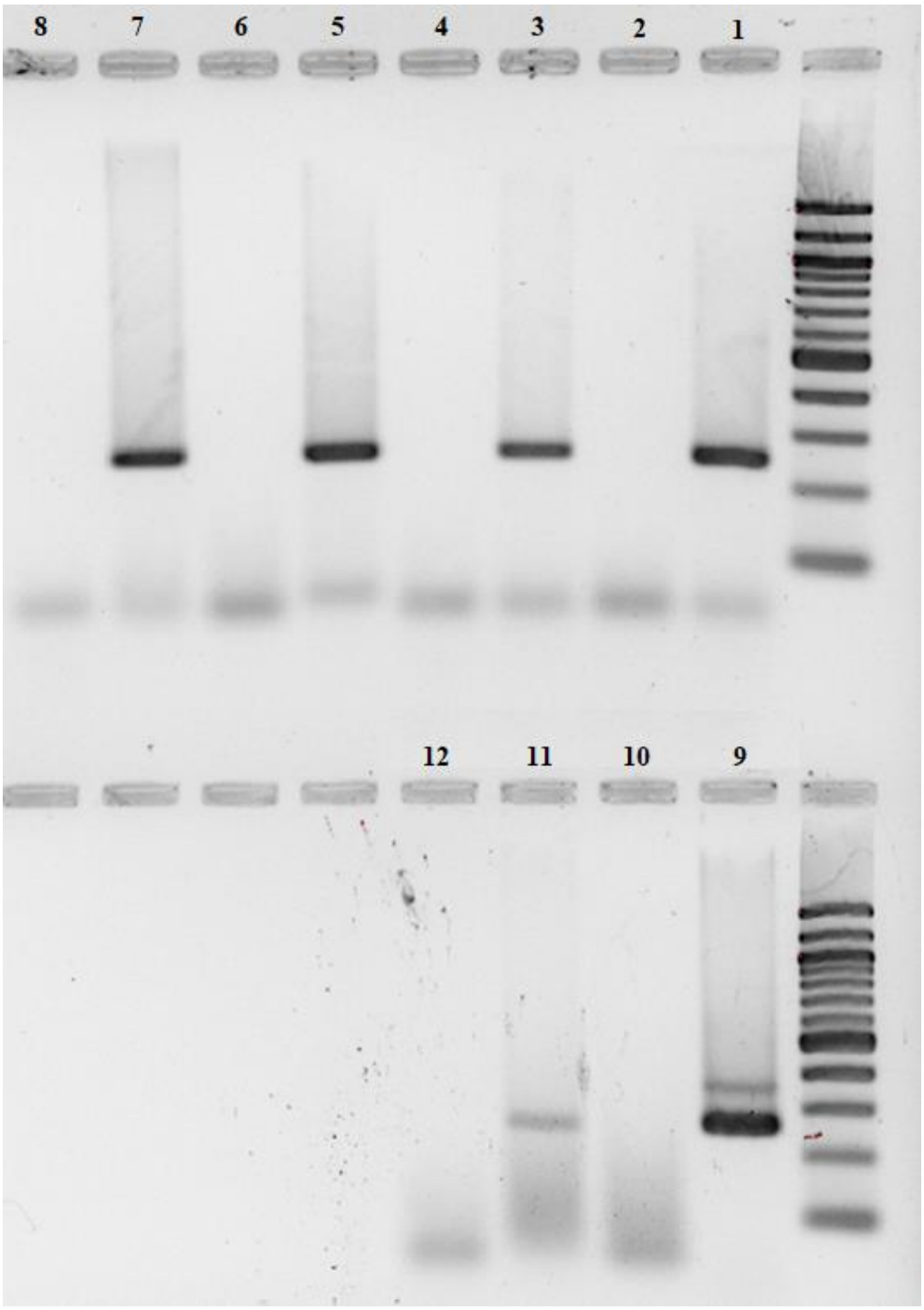

Figure 10: *SpTub-b* step-one amplicons from positive genomic DNA controls. Gel shows alternating positive and negative controls on an annealing temperature gradient (1=genomic DNA 48.2˚C; 2=negative control 48.2˚C; 3=genomic DNA 50.4˚C; 4=negative control 50.4˚C; 5=genomic DNA 53˚C; 6=negative control 53˚C; 7=genomic DNA 55.8˚C; 8=negative control 55.8˚C; 9=genomic DNA 58.5˚C; 10=negative control 58.5˚C; 11=genomic DNA 61˚C; 12=negative control 61˚C). A 100bp ladder is shown to the far right of each row.

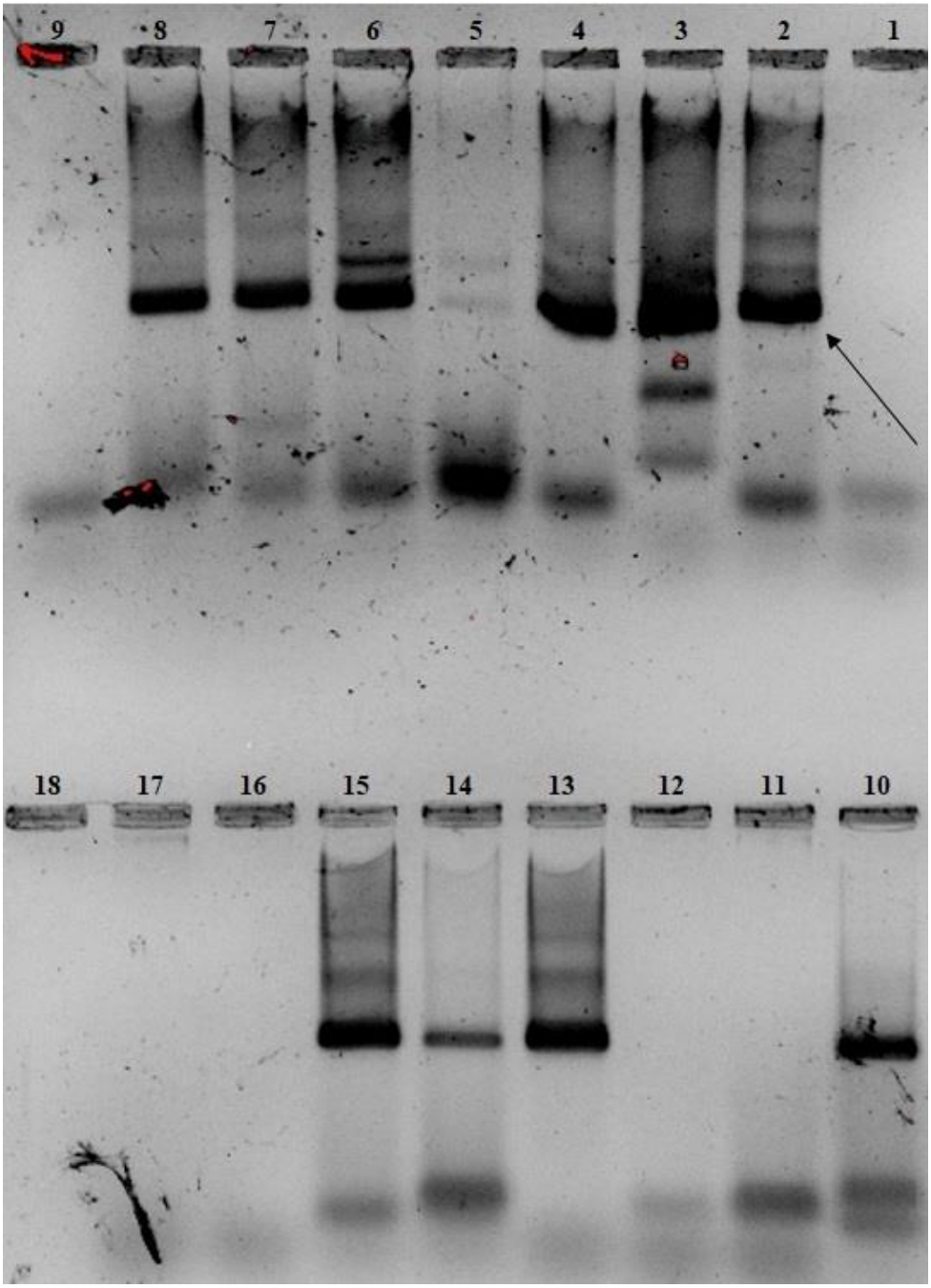

Figure 11: Results from amplifying *SpTub-b* from cDNA template with the same conditions as genomic DNA (1=1118-1 (HAT); 2=1118-1 (HPT); 3=1118-2 (HAT); 4=1118-2 (HPT); 5=1118-3 (HAT); 6=1118-3 (HPT); 7=1118-4 (HAT); 8=1118-4 (HPT); 9=negative control; 10=1118-1 (HAT); 11=1118-1 (HPT); 12=1118-2 (HAT); 13=1118-2 (HPT); 14=1118-3 (HAT); 15=1118-3 (HPT); 16=1118-4 (HAT); 17=1118-4 (HPT); 18=negative control). Non-specific binding is evident. Desired amplicons are approximately 250bp in length; an example of a correctly sized band is indicated with an arrow. [NOTE: top and bottom show different cDNA samples made from the same RNA; only those that amplified were subsequently used.]

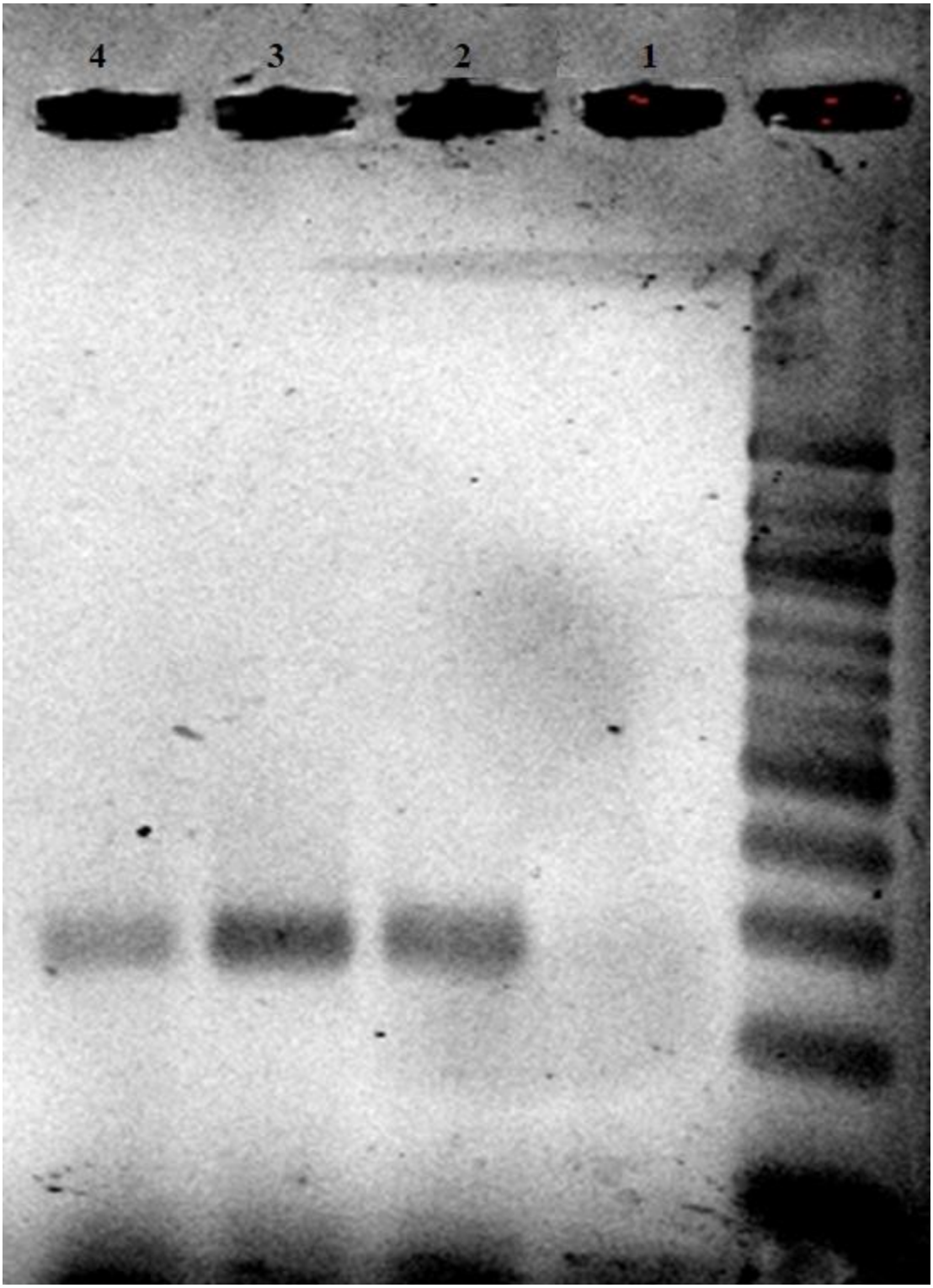

Figure 12: Results from amplifying pooled cDNA with altered conditions (1=negative control; 2=pooled HPT cDNA; 3=pooled HAT DNA; 4=genomic DNA). A slightly distorted 100bp ladder is shown on the far right.

Step-one amplicons were effective template for step-two primers (Figure 13).

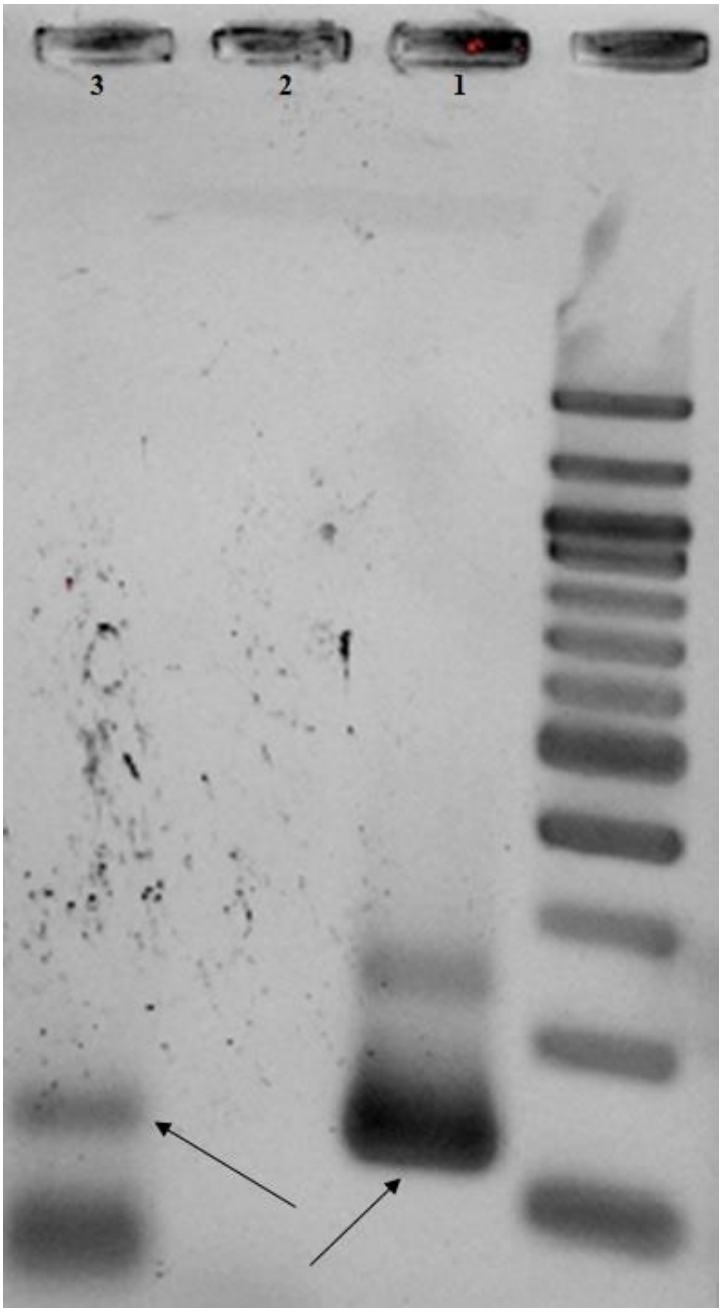

Figure 13: Step-two amplicons of *SpTub-b* (1=genomic DNA; 2=negative control; 3=pooled cDNA). A 100bp ladder is shown on the far right. Image cropped from an annealing temperature gradient gel (ideal annealing temperature of 55.8˚C shown). Top band (~250bp) in well 1 is likely a residual step-one amplicon (this occurs if step-one amplicons are highly concentrated (Snounou and Singh, 2002)). Correctly sized amplicon bands indicated with arrows.

Summary

Overall, the *SPRG_13235* and *SpTub-b* nesting protocols were successful with both genomic DNA and cDNA, while the *SpHtp1* nesting protocol was unsuccessful, necessitating use of step-two primers without nesting (Table 6).

Table 6: Summary of primer design results (0=no apparent amplification; 1=specific amplification).

Target gene	Step-one primers ~genomic DNA~	Step-one primers ~cDNA~	Nesting protocol ~genomic DNA~	Nesting protocol ~cDNA~	Step-two primers ~not nested~
SPRG_13235	1	0	1	1	N/A
SpHtp1	N/A	0	N/A	0	Ambiguous
SpTub-b	1	1	1	1	N/A

Qualitative gene expression and sequence results

SPRG_13235

Although no samples showed amplification of *SPRG_13235* with step-one primers, the gene was amplified from all cDNA samples with nesting protocols (band intensity varied) (Figure 14). Sequencing and BLAST analysis of purified PCR amplicons confirmed that the primers amplified the correct gene (Figure 15). Furthermore, BLAST analysis revealed no other close matches.

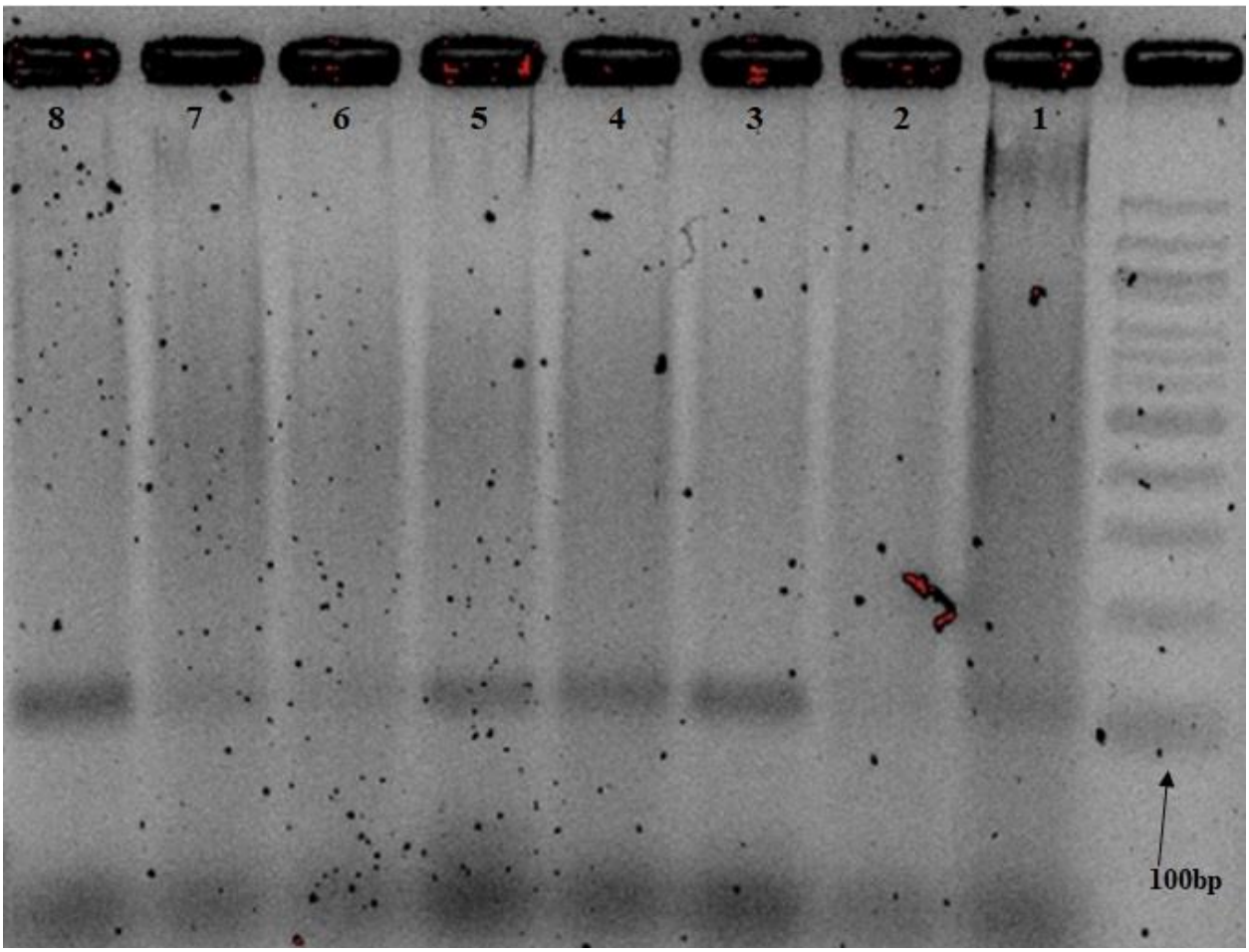

Figure 14: 2.0% agarose gel showing step-two PCR amplicons of *SPRG_13235* (1=1118-1 (HAT); 2=1118-1 (HPT); 3=1118-2 (HAT); 4=1118-2 (HPT); 5=1118-3 (HAT); 6=1118-3 (HPT); 7=1118-4 (HAT); 8=1118-4 (HPT)). A 100bp ladder is shown on the far right (amplicons are slightly larger than 100bp (~112bp)). Separately run negative controls did not show amplification.

GAAGCTGCTCTGGGTTTGCGTCGCGGCCAATGCGCTCGGGCTCTCGCT
TGGCATGGACCTTCTGGCATCCGAA
GAAGCTGCTCTGGGTTTGCGTCGCGGCCAACGCGATCGGGCTCTCGCT
CGGCATGGACCTTCTGGCATCCGAA

Figure 15: *SPRG_13235* step-two primer cDNA amplicon sequence results. cDNA amplicon sequence fragment (bold) is compared to the same theoretical fragment (non-bold). Differences between the theoretical and observed sequence are highlighted in gray.

SpHtp1

Although pooled cDNA showed amplification of *SpHtp1*, individual cDNA samples did not (Figure 16), even when identical conditions were used. Smearing also occurred in these replicates. Sequence data was unclear, suggesting that non-specific amplification occurred, even when only one product size appeared on the gel. Thus, I cannot confidently conclude that *SpHtp1* was expressed in any experimental replicate.

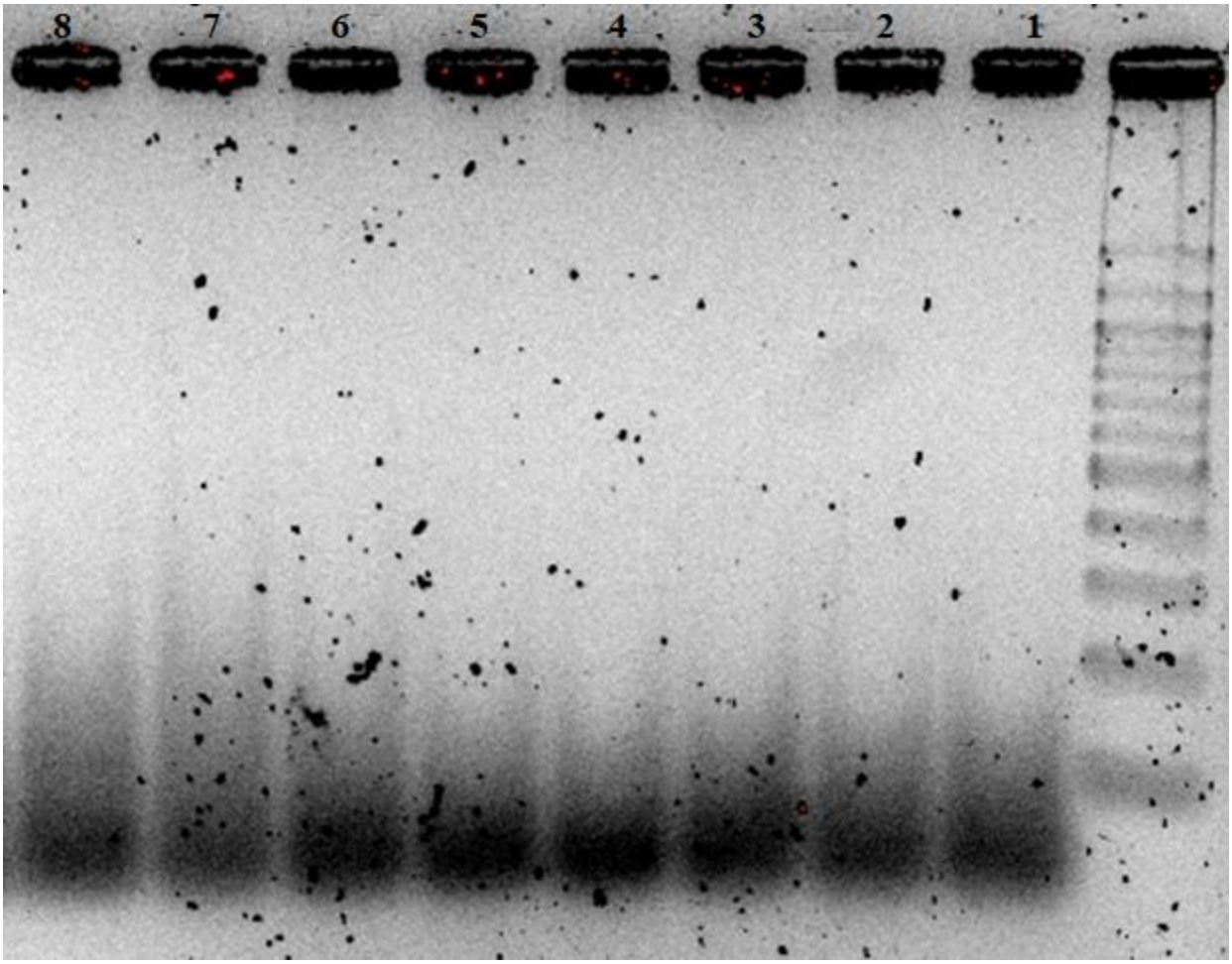

Figure 16: 2.0% agarose gel showing step-two PCR amplicons of *SpHtp1* (1=1118-1 (HAT); 2=1118-1 (HPT); 3=1118-2 (HAT); 4=1118-2 (HPT); 5=1118-3 (HAT); 6=1118-3 (HPT); 7=1118-4 (HAT); 8=1118-4 (HPT)). A 100bp ladder is shown. Smearing was evident on separately run negative controls.

I also tried using the amplicons from the step-two *SpHtp1* primers as template for the same primer set. This method showed that one culture (1118-4 (HAT)) may have expressed *SpHtp1*, as a weak band of approximately the correct size was visible when amplicons were visualized (Figure 17).

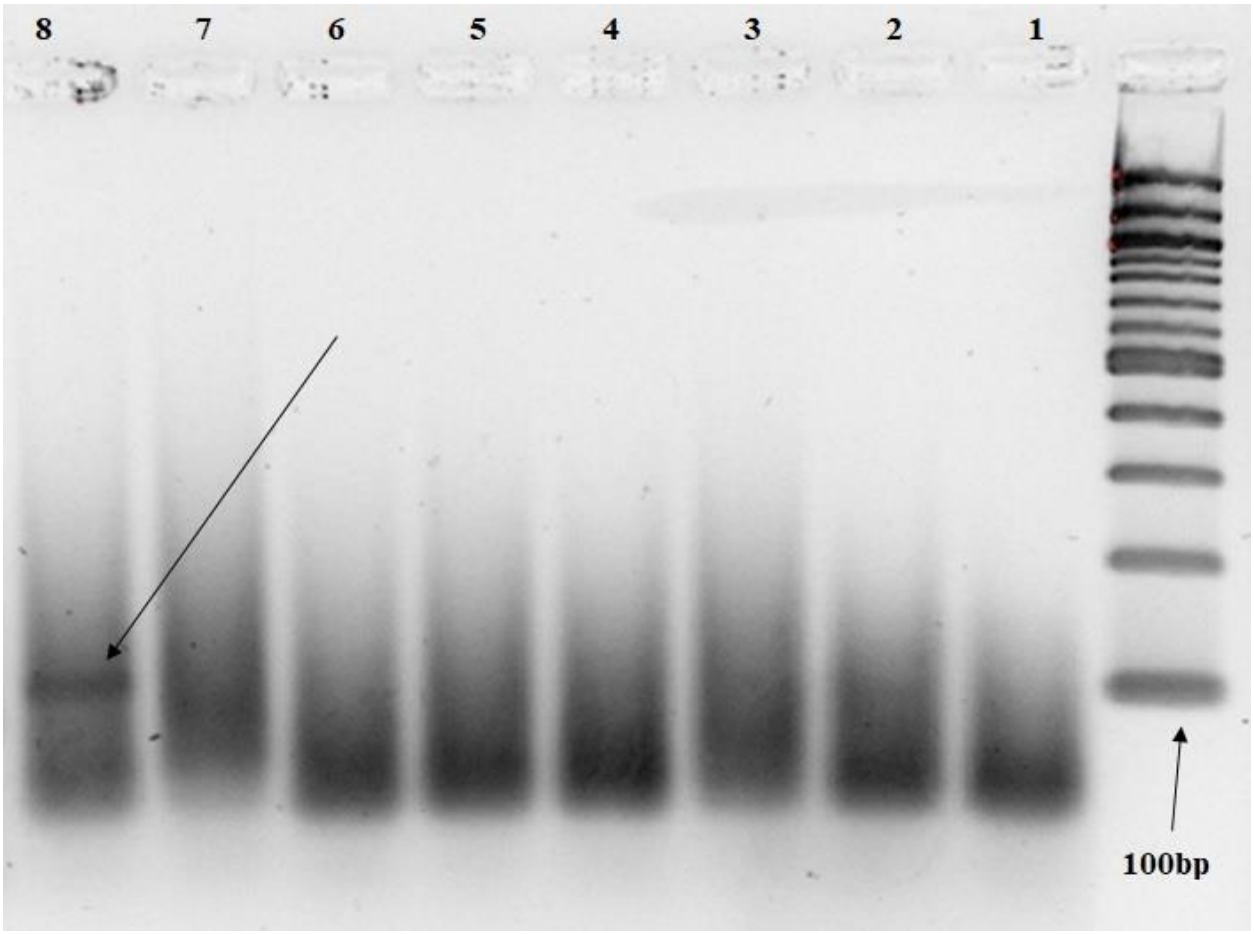

Figure 17: 3.0% agarose gel showing amplification of *SpHtp1* from using step-two *SpHtp1* primers in two successive reactions (1=1118-1 (HAT); 2=1118-1 (HPT); 3=1118-2 (HAT); 4=1118-2 (HPT); 5=1118-3 (HAT); 6=1118-3 (HPT); 7=1118-4 (HAT); 8=1118-4 (HPT)). Separately run negative control showed similar smearing. The desired product size (well 8) is indicated by an arrow.

SpTub-b

SpTub-b was expressed by all experimental and control treatment samples (Figure 11). BLAST analysis of sequenced amplicons confirmed that the correct gene was amplified (99-100% similarity) in both genomic DNA and cDNA. I did not need to use nesting protocols for individual cDNA samples. The step-one amplicon sequence (cDNA) is shown alongside the target sequence in Figure 18.

ACGACGTTCATCGGTAACTCGACGGCGATCCAGGAGATGTTCAAGCGC
GTCTCGGAGCAGTTCACGGCCATGTTCCGGCGGAAGGCTTTCTTGCAT
TGGTACACGGGCGAAGGCATGGACGAGATGGAGTTCACGGAAGCCGAG
TCCAACATGAACGATCTCGTCTCGGAGTACCAGCAGTACCA

ACGACGTTCATCGGTAACTCGACGGCGATCCAGGAGATGTTCAAGCGC
GTCTCGGAGCAGTTCACGGCCATGTTCCGGCGGAAGGCTTTCTTGCAC
TGGTACACGGGCGAAGGCATGGACGAGATGGAGTTTACGGAAGCCGAG
TCCAACATGAACGATCTCGTCTCGGAGTACCAGCAGTACCAA

Figure 18: *SpTub-b* step-one primer cDNA amplicon sequence result. cDNA amplicon sequence fragment (bold) is compared to the same theoretical fragment (non-bold). Differences between the theoretical and observed sequence are highlighted in gray.

Summary of qualitative results

All experimental replicates (both HPT and HAT) expressed *SpTub-b* (β-tubulin gene) and *SPRG_13235* (disintegrin gene) (Table 7). *SpHtp1* expression is unclear (Table 7).

Table 7: Summary of qualitative gene expression results for all cDNA samples (0=expression not detected; 1=expression detected with nesting; 2=expression detected without nesting; 3=expression unclear). At least one sample may have expressed *SpHtp1*.

	cDNA sample							
Gene	**1118-1 HAT**	**1118-1 HPT**	**1118-2 HAT**	**1118-2 HPT**	**1118-3 HAT**	**1118-3 HPT**	**1118-4 HAT**	**1118-4 HPT**
SPRG_13235	1	1	1	1	1	1	1	1
SpHtp1	0	0	0	0	0	0	0	3
SpTub-b	2	2	2	2	2	2	2	2

Quantitative results

My qPCR analysis was unsuccessful. Although the correct products were amplified, and the negative controls showed no contamination products or primer dimers when visualized on an agarose gel (qPCR products of *SpTub-b* step-one primers shown in Figure 19), Ct values were homogenous across dilutions and negative controls (~31), preventing standard curve construction.

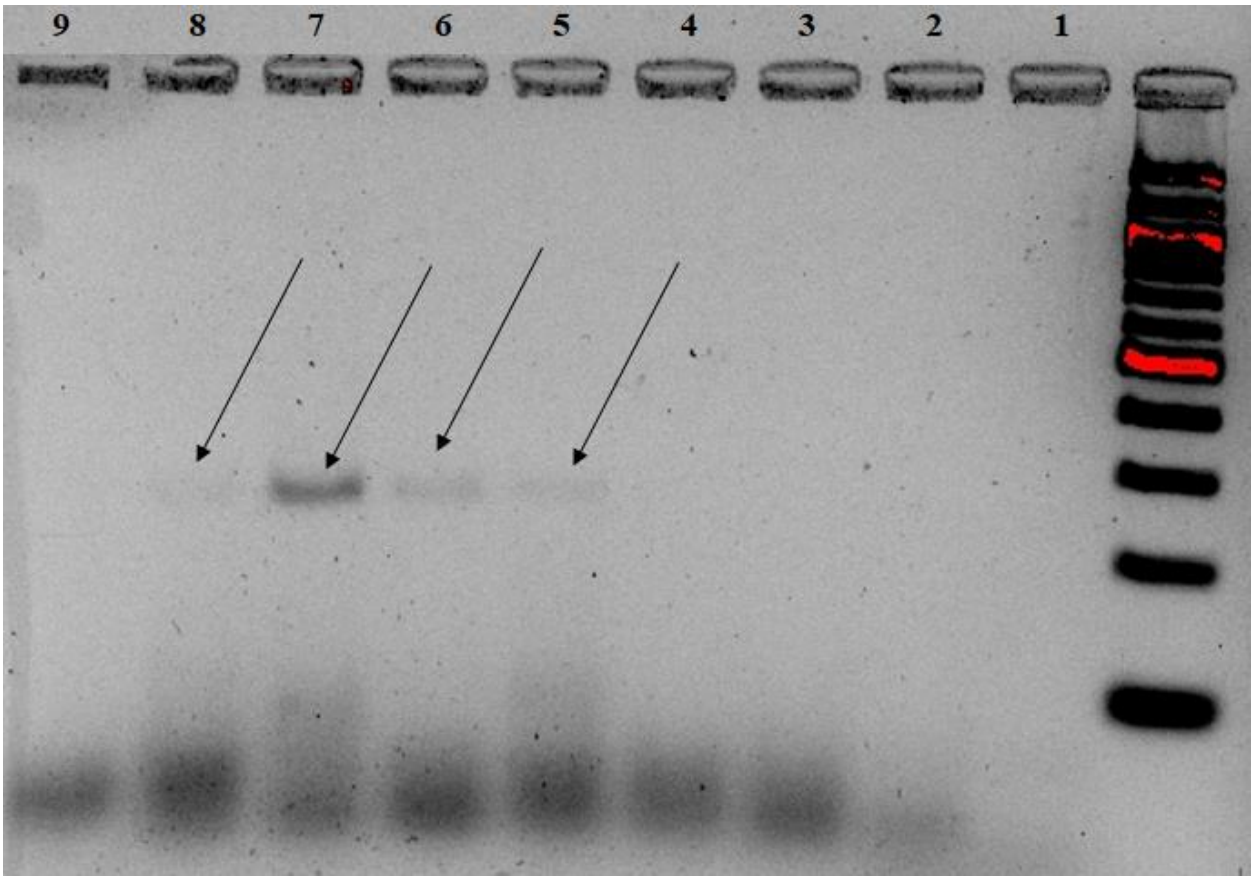

Figure 19: 2.0% agarose gel showing qPCR products from a test of *SpTub-b* step-one primers on genomic DNA and pooled cDNA (1=cDNA ¼ dilution; 2=cDNA ¼ dilution; 3=cDNA ½ dilution; 4=cDNA ½ dilution; 5=undiluted genomic DNA; 6=undiluted genomic DNA; 7=undiluted cDNA, 8=undiluted cDNA; 9=negative control). Band sizes indicating the correct product are marked with arrows. A 100bp ladder is shown on the far right. Band intensity discrepancy between the two undiluted cDNA samples is due to documented pipetting error.

Discussion

Summary of study

I tested the hypothesis that *SPRG_13235* and *SpHtp1* are differentially expressed in host presence and in host absence. However, because my qPCR analysis was unsuccessful, my research neither supports nor refutes this hypothesis. I cannot show that *Saprolegnia parasitica* gene expression varies in saprotrophic and pathogenic circumstances. Nevertheless, my qualitative analysis revealed general *SPRG_13235* expression patterns, and my designed primers may help future researchers to expand on my research and to detect *S. parasitica* in environmental DNA samples.

Disintegrin gene expression

Findings

My research confirms that the putative disintegrin gene *SPRG_13235* is expressed in both host presence and host absence, implying that expression covaries with numerous factors other than host presence (*e.g.* water temperature, nutrient status, or other chemical cues). Expression was low overall, only becoming evident when I used nested PCR protocols. Generally, amplicon band intensity on agarose gels seemed loosely correlated with RNA concentration rather than with treatment; however, I cannot confirm that expression was equivalent across treatments because my qPCR analysis was unsuccessful.

In *S. parasitica*, disintegrins are linked to cyst stages (Jiang et al., 2013; Srivastava et al., 2018). Both expression of disintegrin genes (Jiang et al., 2013) and enrichment of disintegrin proteins (Srivastava et al., 2018) has been confirmed during cyst stages. However, no researchers have evaluated disintegrin gene expression in zoospores. Since my experimental cultures were

theoretically composed mainly of zoospores (with a smaller population of cysts, germinating cysts, and mycelial fragments), my results may suggest that zoospores also express *SPRG_13235*. An alternate interpretation is that only cysts in my cultures expressed *SPRG_13235*.

Potential significance of disintegrin proteins and future directions

Disintegrin genes may have originated via HGT from proteobacteria (Jiang et al., 2013). This HGT event may have occurred before *Saprolegnia* species diverged, as *Saprolegnia diclina* also has putative disintegrin genes. Because *S. parasitica* has a larger, more diversified disintegrin family than *S. diclina*, comparing disintegrin gene expression in these two species may reveal the significance of gene diversification in *S. parasitica*. Currently, nothing is known about how disintegrins function in *S. parasitica*.

Many fish hosts primarily defend against *S. parasitica* with external, nonspecific factors such as antimicrobial compounds in epidermal mucus or mucus-associated bacteria (Wood et al., 1988; Hussein and Hatai, 2001; Carbajal-González et al., 2013). Disrupting the mucus layer exacerbates infections (Hussein and Hatai, 2002). Some hosts also recognize the distinctive oomycete cell wall chemical composition (pathogen-associated molecular pattern: PAMP) and rapidly mount an inflammatory response (Alvarez et al., 1988; Roberge et al., 2007; Belmonte et al., 2014). Still, inflammation does not always occur, implying that immune response varies in different environments (Bly and Clem, 1992; Bly et al., 1993) and in different hosts (Hatai and Hoshiai, 1992; Fregeneda-Grandes at al., 2009). Furthermore, although some hosts produce antibodies against *S. parasitica* (Fregeneda-Grandes at al., 2007, 2009; Minor et al., 2014), inflammation is not always followed by an adaptive immune response, suggesting that *S.*

parasitica can rapidly suppress the host immune system (Fregeneda-Grandes at al., 2009; Belmonte et al., 2014).

Disintegrins may facilitate immunosuppression. Although most previous studies on viper venom disintegrins focus on how disintegrins prevent platelet aggregation by inhibiting fibrinogen binding to platelets (Paine et al., 1992; Kamiguti et al., 1998; Gálan et al., 2008), distintegrins potentially affect many vital integrin functions (Kamiguti et al., 1998; Barja-Fidalgo et al., 2005; Hammouda et al., 2016).

Integrins mediate many vertebrate immune functions (Cannons et al., 2010). First, integrins facilitate cell migration (Tanaka et al., 1993; Selistre-de-Araujo et al., 2010). Leukocytes use integrins to adhere to endothelial cell adhesion molecules, which allows them to transition between tissues (diapedesis) (Shimizu et al., 1990; Tanaka et al., 1993; Cannons et al., 2010). Many immune responses (*e.g.* inflammation) require diapedesis, so disintegrin-mediated integrin blocking may profoundly affect host response (Barja-Fidalgo et al., 2005). Second, integrins maintain immune responses (Shimizu et al., 1990; Cannons et al., 2010). Integrins may activate and deactivate different leukocyte functions, such as movement, respiratory burst, adhesion, cytokine expression, binding, and apoptosis (Barja-Fidalgo et al., 2005). Third, integrins mediate T-cell activation, movement, and attachment (Shimizu et al., 1990; Hogg et al., 2003; Pribilia et al., 2004). For example, T-cells use integrins to adhere to antigen presenting cells (Shimizu et al., 1990; Pribilia et al., 2004) and to interact with B-lymphocytes and dendritic cells (Barja-Fidalgo et al., 2005). Overall, evaluating how disintegrins alter host immune response may be an important next step to understanding how *S. parasitica* modulates host response; disintegrin-mediated alterations of the above processes would greatly reduce the potential host's ability to combat infection.

***SpHtp1* expression**

Findings

I did not conclusively determine which experimental samples expressed *SpHtp1*, as my nesting protocol was unsuccessful, and individual cDNA samples did not show amplification without nesting. This suggests either that the primers worked poorly or that expression did not occur. Amplification patterns appeared random when I used annealing temperature gradients to optimize primers (Figure 8), suggesting that the primers failed to work effectively. Furthermore, DNA sequences were unclear, suggesting non-specific binding.

Previously, van West et al. (2010) found that *S. parasitica* zoospores expressed *SpHtp1* immediately after exposure to host cell lines (<4 hours). However, *SpHtp1* is expressed in lower quantities in cysts, germinating cysts, and sporulating mycelia (van West et al., 2010). Likely, I failed to detect expression due to primer issues; my primers did not amplify high-quality genomic DNA, suggesting that expression was not the main issue.

Potential significance of SpHtp1 and future directions

Oomycete RXLR and RXLR-like effectors are vital in host infection processes. However, excepting the RXLR motif, few functional characteristics unify RXLR effectors; thus, each protein must be separately evaluated. Diverse functions exist. For example, one RXLR effector in *Phytophthora sojae* has a Nudix-like domain with hydrolase activity (gene upregulated in cysts and germinating cysts) (Dong et al., 2011), while *Hyaloperonospora arabidopsidis* uses one of its RXLR effectors to suppress callose deposition, an important aspect of its host's response to PAMPs (Fabro et al., 2011). Currently, nothing is known about how *SpHtp1* directly influences its hosts or how potential host resistance impacts the infection

process. Host recognition of peronosporalean RXLR effectors facilitates host response to infection (Wawra et al., 2012b). Therefore, host recognition of *SpHtp1* may limit the host range of *S. parasitica*. Evaluating the evolutionary origins of RXLR effectors may also reveal functional relationships between RXLR effectors in plant and animal pathogenic oomycetes.

Primer optimization

Effectiveness of nesting

Theoretically, nested PCR improves the likelihood of amplifying the correct DNA sequence because non-specific binding is highly unlikely in the second step, even when cycling conditions are not stringent. Additionally, nested PCR protocols allow researchers to detect small DNA quantities. Thus, nesting has numerous applications. For example, nesting is frequently used to detect the presence of parasites, such as *Plasmodium*, that severely impact populations even in low abundance (Snounou et al., 1993; Snounou and Singh, 2002) or to evaluate microbial community composition in low concentration environmental DNA samples (Oros-Sichler et al., 2006).

In my study, I used nesting protocols both to increase primer specificity and to detect low gene expression levels. Two of the three nesting protocols I designed were highly effective and may aid future researchers.

First, the *SPRG_13235* nesting protocol allowed me both to detect gene expression in low concentration, impure cDNA and to amplify low concentration (<0.15ng/µL) genomic DNA template. In fact, sequenced amplicons showed BLAST similarity only to *SPRG_13235* and not to any other gene in any other organism. Thus, the designed protocol may be used as a species-specific diagnostic tool on DNA extracted from water samples. Nevertheless, this protocol

should first be tested with template DNA from both closely and distantly related species from the order Saprolegniales to confirm specificity.

Second, although *SpTub-b* expression was apparent without nesting, the *SpTub-b* nesting protocol worked effectively. Because *S. parasitica* always expresses *SpTub-b*, future researchers can use this protocol to assess RNA and cDNA quality. Lack of amplification with this primer set may indicate that either RNA extraction or reverse transcription was unsuccessful. Testing this protocol with serially diluted samples of known concentrations will reveal its sensitivity.

The *SpHtp1* nesting protocol was unsuccessful when tested with both genomic DNA and cDNA templates. Step-one primers produced excessive non-specific binding, and when step-one amplicons were used as template for step-two primers, the desired product failed to amplify. PCR additives, such as DMSO and BSA did not change this result, nor did altered cycling conditions. The non-specific nature of the step-one primer set may stem from the repetitive nature of the target sequence. In the future, designing a nested protocol in a different region may prove more effective.

Using step-two primers in isolation appeared to work initially, but I was unable to replicate the results. Additionally, annealing temperature gradients did not show directional improvement as annealing temperatures increased or decreased. In fact, annealing temperature randomly affected amplicon appearance, and some negative controls showed amplification (Figure 9). Negative control amplification disappeared at higher annealing temperatures.

Potential sources of error and study improvements

qPCR difficulties

I was unable to conclusively determine why my qPCR analysis was unsuccessful. My three main problems were: uniform Ct values across serial dilutions, amplification in negative controls, and potential measuring problems. I will summarize potential causes of these problems below.

Uniform Ct values

Uniform Ct values across serial dilutions likely resulted from either low primer efficiency or low cDNA template concentration and quality. Because I optimized primers before performing qPCR, template problems likely predominated.

First, when template concentration is low, stochastic processes partially determine the true concentration of dilutions, since template distribution in the original sample may not be uniform. This causes incorrect Ct values. I initially tried to remedy this by using serial dilutions of step-one amplicons from my standard PCR as my qPCR template (nested qPCR); theoretically, this should have substantially increased template concentration. However, nested qPCR resulted in unusually low Ct values for undiluted step-one amplicons (SYBR green dye likely adhered to already present double-stranded DNA) and uniform Ct values when step-one amplicons were diluted.

Second, low template purity likely contributed to poor qPCR results. Most extracted RNA samples were impure. Effects of impurity were minimized in nested PCR protocols but likely impacted qPCR. Attempts to purify RNA were unsuccessful.

Third, qualitative analyses revealed that expression was low in all replicates, and Figure 21 shows that only undiluted cDNA and genomic DNA templates showed visible amplification of the desired genes when visualized on an agarose gel. Successful nested qPCR would have solved this problem.

I also troubleshot reaction conditions to see if primer efficiency caused problems. First, I evaluated primer concentrations from 50nM to 300nM. Second, I systematically altered annealing temperatures, annealing times, and extension times. Neither strategy worked, and identical problems persisted with each tested primer set. (Note: the step-two primer sets for both *SpHtp1* and *SpTub-b* worked effectively when used in qPCR by van West et al. (2010).)

Amplification in negative controls

Amplification in negative controls persisted regardless of general precautionary measures against contamination. Thus, I tried four additional strategies. First, I prepared negative and positive controls in separate strip-tubes. Second, I mixed and closed my negative control strip-tubes before opening DNA samples. Third, I replaced all reagents and plastic-ware to minimize contamination. Fourth, I adjusted cycling conditions to eliminate potential primer dimers (*i.e.* decreased annealing times and increased annealing temperatures). These strategies were unsuccessful.

Measuring problems

Visualizing qPCR products on a 2.0% agarose gel revealed that the correct products were amplified by qPCR, that undiluted and diluted samples showed differences in product band intensity, and that negative controls showed no amplification (Figure 19). This suggests a

potential machine error, but because other people have successfully used the same machine, this is unlikely.

RNA extractions

Improving RNA extraction procedures would have increased my study's explanatory power by increasing RNA concentrations and improving RNA purity. Although I used 75mL of liquid zoospore culture in each experiment, all RNA samples had low concentrations. Lack of obvious pellet formation made it difficult to gauge whether subsequent steps were performed effectively. For example, I could see neither pellet adhesion nor suspension. In the future, I would use more liquid zoospore culture in each experiment to facilitate pellet formation. I would also confirm RNA presence using gel electrophoresis before converting RNA to cDNA.

Life stage control

I normalized the confounding impact of life stage by using a paired experimental design. Nevertheless, *Saprolegnia* develops rapidly, so determining each culture's exact life stage composition is difficult. I assumed that each culture contained a mixture of zoospores, cysts, germinating cysts, and mycelial fragments but could not determine exact quantities or proportions. Moreover, mechanical stimuli, such as vortexing, can induce encystment (Andersson and Cerenius, 2002), so vigorously mixing cultures with swirling prior to experiments may have induced encystment. Furthermore, remaining zoospores (or zoospores formed during experiments via polyplanetism) may have encysted when centrifuged prior to RNA extractions, confounding the impact of host presence and equalizing expression in all

samples. Testing how different cell harvesting techniques impact apparent expression levels may confirm or refute this possibility.

Because my study only evaluated the effect of host presence, these uncertainties did not necessarily introduce biases. In the future, however, I would extract RNA at different time points to examine interacting effects of host presence and life stage. For example, host presence may not influence mycelial gene expression, since gene expression at this stage primarily involves maintenance of primary metabolism and hyphal growth (Srivastava et al., 2018). Contrarily, host presence may profoundly affect gene expression in cysts, which express many virulence genes (Jiang et al., 2013; Srivastava et al., 2018).

Host effluents

Saprolegnia parasitica zoospores exhibit chemotaxis (Walker and van West, 2007). Thus, host effluents in experimental water samples likely provided a strong signal. Nevertheless, comparing how isolated host effluents and living hosts differentially impact zoospores may reveal interesting patterns. Furthermore, additional host absence treatments may more accurately show how *S. parasitica* expresses genes when acting as a saprotroph. For example, dead fish tissue or plant substrates could be added as potential nutrient sources. A lack of available nutrients may have simply induced polyplanetism.

Future Research

Context dependency

The effect of oomycete life stage on gene expression is well documented. However, few researchers have evaluated how gene expression in *S. parasitica* varies along biotic or abiotic

environmental gradients. Since *S. parasitica* is a facultative pathogen, its gene expression patterns likely change when it is exposed to certain environmental cues. My study evaluated changes in gene expression based on the presence of general host effluents, but certain chemicals may be more important than others. Future researcher should evaluate which host-derived chemicals affect *S. parasitica* gene expression.

Other potential virulence genes

Although many researchers over the past fifty years have qualitatively analyzed infection processes in *S. parasitica*, molecular mechanisms are largely unknown. As a hemibiotroph, *Saprolegnia parasitica* has an initial biotrophic stage (Jiang et al., 2013) in which it escapes host defences without eliciting an adaptive immune response (Belmonte et al., 2014) followed by a necrotrophic stage in which it kills host tissues (Peduzzi and Bizzozero, 1977). Few authors have evaluated the molecular mechanisms behind the observed host-pathogen relationship.

First, researchers should deeply evaluate how *S. parasitica* suppresses the host immune system. Although disintegrins and SpHtp1 likely contribute, other proteins, such as haemolysins, may also be important. Second, researchers should characterize the molecular mechanisms behind the biotrophy-necrotrophy switch. Many authors have observed this infection pattern qualitatively, but few details are available. During the necrotrophic stage, *S. parasitica* may secrete toxins and enzymes that allow it to penetrate deeper into host tissues (Peduzzi and Bizzozero, 1977). Thus, genes expressed in zoospores, cysts, and germinating cysts may facilitate biotrophy, while genes expressed in later mycelial stages may facilitate necrotrophy and deeper penetration of host tissue. Identifying which genes are expressed at different infection

stages and in different environments will reveal both the underlying reasons for certain host responses and the context-dependency of saprolegniasis.

Host specificity

S. parasitica host specificity should be further evaluated. Tiffney's (1939) paper revealed a shockingly large teleost host range and showed that salamanders and frogs are vulnerable. More recent studies show that *S. parasitica* infects invertebrates such as crayfish (Diéguez-Uribeondo et al., 1994) and insect larvae (Wuensch et al., 2018). Most research has historically focused on fish hosts because saprolegniasis pervades aquaculture facilities.

Amphibians are extremely sensitive to environmental stresses, such as pollution, and are highly vulnerable to disease (Lefcort et al., 1997; Kiesecker et al., 2001a, 2001b). Therefore, as environmental stresses increase in severity, amphibian saprolegniasis may become ubiquitous. Furthermore, asymptomatic fish from hatcheries may spread *S. parasitica* inoculum to natural ecosystems if introduced (Kiesecker et al., 2001a, 2001b). Thus, more research should evaluate amphibian susceptibility in natural ecosystems.

Other species in the Order Saprolegniales

The current lack of genetic characterization in the order Saprolegniales prevents researchers from evaluating genetic similarities and differences among species. Several species have had their genomes sequenced, but researchers primarily compare animal pathogens to plant pathogens, only briefly analyzing genes without orthologs. Thus, the unifying characteristics and conserved genes within the order Saprolegniales are unknown. Overall, future *S. parasitica* research should evaluate infection strategies by comparing groups of related animal pathogens.

Comparative studies may reveal interesting patterns. Although *S. parasitica* is hemibiotrophic, other species, such as *S. diclina* and *Aphanomyces invadans*, are opportunistic necrotrophs (Oidtmann, 2011; Anderson et al., 2015; Songe et al., 2016). Analyzing the genome and transcriptome of these potential necrotrophs may expose interesting differences and reveal possible evolutionary divergence patterns (*e.g.* was the common ancestor of oomycete animal pathogens saprotrophic or parasitic?). Comparing *S. parasitica* to *A. invadans* is particularly crucial, as these species have comparable impacts on their fish hosts and may interact with each other in aquaculture settings (Oidtmann, 2011).

Conclusion

I cannot conclude that *S. parasitica* virulence gene expression differs in host presence and host absence. However, my analysis revealed that *S. parasitica* expresses *SPRG_13235* in both host presence and host absence, implying that expression is not solely determined by host presence but likely co-depends on factors such as life stage, nutrient status, and abiotic environment. Further research into these factors may reveal how facultative pathogens find niches. Additionally, my study demonstrates that low *S. parasitica* gene expression can be detected using nested PCR protocols. This has numerous applications in both experimental and observational *S. parasitica* research, and my designed primers can be used by future researchers.

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