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Assessment of intra-specific variability in *Saprolegnia parasitica* populations of aquaculture facilities in British Columbia, Canada

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ABSTRACT: Among the *Saprolegnia* species found in aquaculture facilities, *S. parasitica* is recognized as the primary fish pathogen and remains an ongoing concern in fish health management. Until recently, these pathogens were kept in check by use of malachite green; due to its toxicity, this chemical has now been banned from use in many countries. It is difficult to predict and control *S. parasitica* outbreaks in freshwater systems and there is a need to understand the population genetic structure of this pathogen. Genetic characterization of this species in aquaculture systems would provide information to track introductions and determine possible sources of inoculum. Degenerate PCR primers containing short sequence repeats were used to create microsatellite-associated genetic markers (random amplified microsatellites) for the comparison of *S. parasitica* isolates collected primarily from commercial Atlantic salmon aquaculture systems in British Columbia, Canada, over a 15 mo period to describe their spatial and temporal variability. The frequencies of amplified products were compared and the population genetic diversity was measured using Nei's genetic distance and Shannon's information index, while the species population structure was evaluated by phylogenetic analysis. *S. parasitica* was detected in all facilities sampled. Genetic diversity was low but not clonal, most likely due to repeated introduction events and a low level of sexual recombination over time. A better understanding of pathogen population structure will assist the development of effective preventative measures and targeted treatments for disease outbreaks.

KEY WORDS: Oomycete · *Salmo salar* · Saprolegniosis · Microsatellites · Aquaculture

INTRODUCTION

Water molds of the genus *Saprolegnia* are eukaryotes of the class Oomycota, phylum Heterokontophyta. These aquatic organisms have a wide geographical distribution in freshwater ecosystems where they exist as saprophytes and, in some cases, as opportunistic animal pathogens that may infect a wide range of vertebrate species. In recent decades, they have been identified as the causal agent of a persistent fish health problem (saprolegniosis) in both enhancement hatcheries and commercial aquaculture facilities, causing poor recruitment and reduced productivity for a number of fish species. This disease can contribute to fish mortality, depending on the severity of infection, the initial health status of the fish, and other factors (van West 2006, Phillips et al. 2008, Robertson et al. 2009).

The taxonomy of this genus has been clarified in recent years in an effort to define the specific taxa responsible for disease outbreaks in freshwater fish (Sandoval-Sierra et al. 2014, de la Bastide et al. 2015). *S. parasitica* is believed to be the primary causal agent of saprolegniosis, a disease character-

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ized by white or grey patches of filamentous mycelial growth associated with epidermal lesions on infected fish. This species was isolated most frequently in Canadian aquaculture facilities producing Atlantic salmon *Salmo salar* from both system water and fish tissues (de la Bastide et al. 2015); other *Saprolegnia* spp. can often be detected in the same commercial aquaculture systems (e.g. *S. diclina* on eggs), but rarely infect fish (de la Bastide et al. 2016).

With the banning of malachite green as an antimicrobial agent, saprolegniosis has become more prevalent in aquaculture (van West 2006, Robertson et al. 2009), prompting the development of new control measures that demonstrate variable efficacy (Gieseker et al. 2006, Aller-Gancedo & Fregeneda-Grandes 2007, Mitchell et al. 2010, Sun et al. 2014). Despite various efforts to prevent saprolegniosis, many basic aspects of species pathology remain poorly understood. Little is known of the mode(s) of introduction into aquaculture systems, the rate of infection for different host life stages, the factors that affect host susceptibility, whether pathogen virulence varies among isolates, and the genetic diversity of the pathogen in commercial systems. Although the physiology and life cycle of *S. parasitica* have been well described (Willoughby 1985, Beakes et al. 1994, Diéguez-Uribeondo et al. 2007) and infection mechanisms are under study (Jiang et al. 2013, Belmonte et al. 2014, Minor et al. 2014), the population structure of this species is not well understood (Robertson et al. 2009). Information describing intraspecific variability would be useful in understanding this opportunistic pathogen and the factors that contribute to disease development in aquaculture facilities. Genetic characterization of *S. parasitica* from water and colonized tissues will also provide information to track introductions of pathogenic isolates and determine possible sources of inoculum in production systems.

Nucleotide sequence analysis of the internal transcribed spacer (ITS)-rDNA regions may be used to confirm species identity for members of the genus *Saprolegnia* (de la Bastide et al. 2015). In order to describe intra-specific variability, the evaluation of other sequence regions is required, including microsatellites. Also known as simple sequence repeats (SSRs), microsatellites are typically composed of 1–6 nucleotide tandem repeats. They are distributed throughout the genome and are inherited in a Mendelian fashion as codominant markers. Their high polymorphism rates, high reproducibility, and broad distribution have made microsatellites useful as genetic markers for the characterization of both inter- and intra-specific genetic variability (Litt & Luty 1989, Tautz 1989, Charlesworth et al. 1994, Miah et al. 2013). This variability may be assessed at many loci in the target genome, with the requirement of some nucleotide sequence information for oligonucleotide primer design. Here we used similar genetic markers known as random amplified microsatellites (RAMS) to compare isolates of *S. parasitica* (Zietkiewicz et al. 1994, Hantula et al. 1996); these 2 approaches are compared in Fig. 1.

RAMS are microsatellite-associated markers generated using PCR primers that contain both selected SSR sequences and a short length of degenerate nucleotides that anchor the primer at the 5′ end of the SSR. Microsatellite markers are based upon primers that amplify only the SSR and are designed using flanking sequence data to target each specific locus. In contrast, RAMS primers will anneal to any complementary regions and amplify both the targeted repeat and the non-SSR sequences between the 2 primer sites, often at multiple loci. The detection and length of amplified products will be determined by the proximity of the primer sites, the incidence of

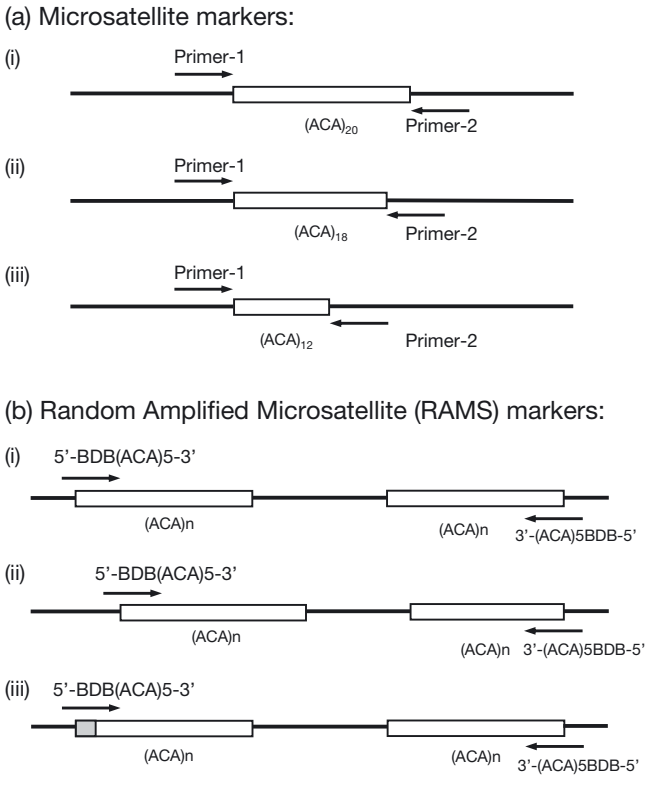

Fig. 1. Comparison of (a) microsatellite and (b) random amplified microsatellite (RAMS) markers. See 'Materials and methods: Primer development for population genetic analysis' for complete description

insertions/deletions between these sites at a given locus, and the conditions of the PCR protocol (Zietkiewicz et al. 1994). The amplification of common doublet and triplet SSR and intervening nucleotide sequences will generate reliable genetic markers that are widely dispersed in the target genome and provide more information about isolate identity than microsatellite markers alone, without the requirement of primer target sequence data. The collection of amplicons obtained with each primer–isolate combination will highlight similarities and differences among studied isolates, thus providing insight into relatedness and population structure.

The population structure of important oomycetous pathogens has been studied using microsatellites and supports our use of RAMS markers to evaluate *S. parasitica*. Genetic variability of the invasive pathogen *Aphanomyces astaci* was clarified using SSR markers to distinguish genotypes associated with different crayfish hosts that vary in their geographical range and susceptibility (Grandjean et al. 2014); these markers assist disease monitoring and management of susceptible European crayfish. In contrast to *S. parasitica*, *A. astaci* appears to display host-specific genotypes. Weiland et al. (2015) examined the genetic diversity of *Pythium* spp. with SSR and amplified fragment length polymorphism (AFLP) markers; these plant pathogens cause significant losses in conifer nurseries, and fungicide-resistant genotypes of 1 species (*P. ultimum*) were traced through the movement of infected nursery stock. The prevalence of these pathogens in geographically separated nursery islands has parallels with *S. parasitica* in freshwater aquaculture facilities. Genotyping tools for the plant pathogen *Phytophthora infestans* were developed using multiplex PCR and 12 SSR marker loci to distinguish genotypes, useful for rapid population analysis and pathogen genotype monitoring in agricultural systems (Li et al. 2013). Fast-evolving microsatellite markers were useful in describing population structure and long-distance migration patterns of the invasive pathogen *Phytophthora ramorum*; clonally reproducing lineages in US nurseries revealed genetic variability within microsatellite regions (Goss et al. 2009).

In the present study, our objective was to assess the genetic diversity of *S. parasitica* isolates among 8 sample locations in British Columbia, and to evaluate whether the population structure varied over a 15 mo sampling interval for a single aquaculture facility that was subject to a more intensive sampling regime.

MATERIALS AND METHODS

Field sample collection and nucleotide sequence analysis

Samples were collected between September 2009 and November 2012 from 8 aquaculture facilities and 1 freshwater site in British Columbia, Canada, to survey the diversity of *Saprolegnia parasitica* genotypes. Isolates were obtained from samples of infected fish, fish eggs, swabs of work surfaces, and water from different areas of a facility. These isolates were cultured, used for DNA extraction, and subjected to nucleotide sequence analysis (ITS-rDNA region) to confirm species identity, according to the methods described by de la Bastide et al. (2015). A subset totaling 87 *S. parasitica* isolates was selected for use in the current study. These included isolates from all sample locations, as well as a larger number of samples from a single aquaculture site (Sayward Hatchery North) that were studied to evaluate genetic variability over time (Table 1).

Primer development for population genetic analysis

As described in Fig. 1, the use of microsatellite markers requires knowledge of the nucleotide sequence in order to design forward (Primer1) and reverse (Primer2) PCR primers that anneal to flanking regions adjacent to the selected microsatellite containing tandem repeats (e.g. ACA) of variable length. Allelic variation for a given locus will be due to variability in the number of tandem repeats, in this instance varying from 20 to 12 copies of the ACA repeat (i, ii, and iii). (b) RAMS markers are associated with microsatellite regions, but use a single degenerate 5′-anchored PCR primer that includes a short tandem repeat and degenerate nucleotides that will anneal to the flanking region adjacent to the 5′-end of the selected tandem repeat sequence (e.g. ACA). Sequences amplified by PCR will include the 2 microsatellites and the intervening non-microsatellite region. Allelic variation will arise as a consequence of length polymorphisms in the amplified regions (i and ii) and non-amplification due to primer mismatches at the annealing site (iii, gray portion of sequence) caused by insertions or deletions.

Primer development initially targeted conventional microsatellite markers in *S. parasitica* and began with the examination of a small expressed sequence tag (EST) library (Torto-Alalibo et al. 2005) available in GenBank (library name: LIBEST_017183 *Sapro-*

Table 1. Sampling sites in British Columbia, Canada, number of *Saprolegnia parasitica* isolates from each sample location used for population analysis, and the source of these isolates

Sample collection site	Code	Location (GPS coordinates)	Number of isolates selected (source)
Nanaimo River Hatchery, private	NR	49.072° N, 123.873° W	1 (egg) 2 (water)
Puntledge River Hatchery, DFO-Canada	PR	49.688° N, 125.036° W	3 (egg)
Sayward Hatchery North, Marine Harvest Canada	SN	50.325° N, 125.920° W	4 (swabs) 31 (fish) 33 (water)
Sayward Hatchery South, Marine Harvest Canada	SS	50.252° N, 125.734° W	4 (fish)
United Hatchery, Marine Harvest Canada	UH	49.404° N, 124.991° W	2 (fish)
Ocean Falls, Marine Harvest Canada	OF	52.354° N, 127.694° W	2 (fish)
Georgie Lake, Marine Harvest Canada	GL	50.749° N, 127.673° W	1 (water) 2 (fish)
Stelling Hatchery, Cermaq Canada	SH	49.489° N, 124.805° W	1 (water)
Upper Goldstream, Goldstream Provincial Park	UG	48.485° N, 123.548° W	1 (water)

legnia parasitica ATCC90214 mycelium). The library contained 1279 consensus EST sequences that were screened for the presence of SSR sequences using the Tandem Repeats Finder software (Benson 1999); a total of 115 EST sequences were determined to contain SSRs, and PCR primer pairs were designed for their amplification. A subset was selected (18 primer sets) to evaluate their utility in distinguishing closely related genotypes of *S. parasitica*.

The evaluation of conventional microsatellite markers did not provide useful results. Consequently, we selected microsatellite-associated markers (RAMS) to assess the genetic diversity of isolates. A total of 7 degenerate, 5′-anchored primers were selected, each targeting a unique SSR sequence. These primers amplify variable-length DNA fragments to generate unique fingerprints for different genotypes. Primer design was based upon those used previously by Hantula et al. (1996) and the approach of Zietkiewicz et al. (1994). Degenerate primer sequences were subsequently modified according to the abundance of SSRs detected in EST libraries compiled in previous studies of both oomycete and fungal genomes that were subject to similar genetic analyses (Van der Nest et al. 2000, Karaoglu et al. 2005, Lee & Moorman 2008).

A series of screening experiments was conducted with existing primers (Hantula et al. 1996) and new degenerate primers developed for genotype comparison to determine their resolving power and to establish which primers might be most informative. Selected primers generated clear and repeatable amplification products, with some variation in the number or size of amplicons between different isolates. Four reference isolates (Table 2, isolate numbers 24, 42, 131, and 306) collected at different geographical locations and different times were used for preliminary tests of primers to maximize the likelihood that genetic marker variability would be observed in the amplification profiles.

From our set of 87 *S. parasitica* isolates (Table 2), an initial group of 46 isolates was selected from our collection for the assessment of their genetic diversity using a random number generator (web resource: www.random.org/). The same selection process was completed for a second group of 46 isolates, with the selective use of 5 isolates from the first group. This approach was dictated by the sample capacity of the large, 50-lane gel apparatus used to separate and score amplification products for the presence or absence of amplicons. The use of 5 shared isolates allowed the valid comparison of amplification profiles for all 87 isolates screened with a given degenerate primer.

Reaction conditions and gel electrophoresis analysis of PCR products

Each PCR reaction was performed in 10 µl final volume using 1 unit of DNA polymerase (Fermentas Dream *Taq*), a final concentration of 0.5 µM for the primer, and 5.0 ng of genomic DNA per reaction. All PCR amplifications used only a single degenerate primer per reaction and were performed using an Eppendorf Mastercycler® Gradient model 5331. Reaction conditions included an initial denaturation step (10 min at 95°C), followed by 35 cycles of de-

Table 2. Isolates assessed for genetic diversity in the current study (isolate number as assigned in the current study). Species designations were assigned subsequent to sequence analysis. Isolate collection date, geographical origin (site ID codes as in Table 1), source material (eggs [e], fish [f], swabs [s], or water [w] and sampling location), and label for phylogenetic analysis (isolate number_sample date_site_source material; see Fig. 3) are indicated. Accession numbers are given (where applicable) for the ITS-rDNA sequence data submitted previously to GenBank for selected isolates (ns: not submitted). Four isolates (24, 42 131, 306) were used for the preliminary screening of PCR primers, while 5 isolates (104, 147, 179, 221, 231) were shared between the 50-lane gels that processed the different isolate collections

Isolate no.	Sample date (dd-mm-yy)	Site	Source material	Label for phylogenetic analysis	Acc. no.
9	22-12-09	NR	e (*Oncorhynchus gorbuscha*)	009_091222_NR_____e	JX212933
24	18-05-10	PR	e (*Oncorhynchus tshawytscha*)	024_100518_PR_____e	JX212936
25	18-05-10	PR	e (*Oncorhynchus tshawytscha*)	025_100518_PR_____e	ns
30	13-05-10	PR	e (*Oncorhynchus tshawytscha*)	030_100513_PR_____e	ns
34	09-11-09	UG	w	034_0911___UG_____w	JX213201
41	22-12-09	NR	w	041_091222_NR_____w	JX213086
42	22-12-09	NR	w, trough	042_091222_NRTroughw	JX213207
63	20-08-10	OF	f (*Salmo salar*)	063_100820_OF_____f	JX213076
66	20-08-10	OF	f (*Salmo salar*)	066_100820_OF_____f	JX213078
72	27-08-10	UH	f (*Salmo salar*)	072_100827_UH_____f	JX213072
81	31-08-10	SN	f (*Salmo salar*), 10M-01	081_100831_SN10M-01f	JX212945
84	31-08-10	SN	w, 10M-01	084_100831_SN10M-01w	JX213209
87	07-09-10	SN	f (*Salmo salar*), 10M-01	087_100907_SN10M-01f	JX213080
88	07-09-10	SN	f (*Salmo salar*), 06M-01	088_100907_SN06M-01f	ns
93	13-09-10	SN	w, 10M-01	093_100913_SN10M-01w	JX213211
96	27-08-10	UH	f (*Salmo salar*)	096_100827_UH_____f	JX212951
98	07-09-10	SN	f (*Salmo salar*), 10M-01	098_100907_SN10M-01f	JX212952
104	20-09-10	SN	f (*Salmo salar*), 06M-05	104_100920_SN06M-05f	JX212957
105	20-09-10	SN	f (*Salmo salar*), 06M-05	105_100920_SN06M-05f	JX212958
107	28-09-10	SN	w, 06M-05	107_100920_SN06M-05w	JX213089
110	28-09-10	SN	f (*Salmo salar*), 06M-05	110_100928_SN06M-05f	JX212961
112	29-09-10	GL	f (*Salmo salar*), penn 3	112_100929_GLPenn3_f	JX212963
114	29-09-10	GL	f (*Salmo salar*), penn 3	114_100929_GLPenn3_f	JX212965
119	28-09-10	SN	w, 10M-04	119_100928_SN10M-04w	JX213090
121	28-09-10	SN	w, 06M-05	121_100928_SN06M-05w	JX213202
123	28-09-10	SN	f (*Salmo salar*), 06M-05	123_100928_SN06M-05f	JX213074
125	29-09-10	GL	w	125_100929_GL_____w	JX213203
131	04-10-10	SN	w, 06M-05	131_101004_SN06M-05w	JX213092
134	04-10-10	SN	f (*Salmo salar*), 06M-05	134_101004_SN06M-05f	JX212967
147	28-09-10	SN	w, 09M-01	147_100928_SN09M-01w	JX213093
161	28-09-10	SN	f (*Salmo salar*), 10M-04	161_100928_SN10M-04f	JX212980
164	20-09-10	SN	f (*Salmo salar*), 10M-04	164_100920_SN10M-04f	JX212987
168	06-10-10	SN	f (*Salmo salar*), Tank 02	168_101006_SHTank02f	JX212988
169	12-10-10	SN	f (*Salmo salar*), 10M-05	169_101012_SN10M-05f	JX212989
171	27-10-10	SN	f (*Salmo salar*), 09M-01	171_101027_SN09M-01f	JX212991
179	12-10-10	SN	f (*Salmo salar*), 06M-05	179_101012_SN06M-05f	JX212992
183	19-10-10	SN	w, anesthetic bath	183_101019_SNSeducrw	JX213116
189	27-10-10	SH	w, tank K7	189_101027_SHK7____w	JX213098
193	12-10-10	SN	w, 09M-01	193_101012_SN09M-01w	JX213099
200	24-11-10	SN	w, pre-treatment	200_101124_SNPretrtw	JX213105
211	24-11-10	SN	s, 10M-04	211_101124_SN10M-04s	JX213117
213	01-12-10	SN	w, hatch tank 24	213_101201_SNHtch24w	JX213118
216	07-12-10	SN	f (*Salmo salar*), 10M-01	216_101207_SN10M-01f	JX212994
218	07-12-10	SN	w, 10M-01	218_101207_SN10M-01w	JX213120
219	07-12-10	SN	w, 10M-01	219_101207_SN10M-01w	JX213121
221	07-12-10	SN	f (*Salmo salar*), 09M-03	221_101207_SN09M-03f	JX212996
231	07-12-10	SN	f (*Salmo salar*), U-03	231_101207_SNU-03__f	JX212999
232	07-12-10	SN	w, U-03	232_101207_SNU-03__w	JX213124
240	07-12-10	SN	w, 06M-11	240_101207_SN06M-11w	JX213126
244	07-12-10	SN	w, U-03	244_101207_SNU-03__w	ns
253	07-12-10	SN	w, 09M-03	253_101207_SN09M-03w	JX213129
256	13-12-10	SN	w, U-sump	256_101213_SNU-sumpw	JX213131

Table continued on next page

Table 2 (continued)

Isolate no.	Sample date (dd-mm-yy)	Site	Source material	Label for phylogenetic analysis	Acc. no.
264	20-12-10	SN	s, vaccination tank	264_101220_SNVacc__s	JX213133
279	11-01-11	SN	f (*Salmo salar*), 06M-09	279_110111_SN06M-09f	JX213003
293	11-01-11	SN	w, 10M-01	293_110111_SN10M-01w	JX213139
303	19-01-11	SN	w, 09M-02	303_110119_SN09M-02w	JX213142
306	19-01-11	SN	f (*Salmo salar*), 10M-01	306_110119_SS10M-01f	JX213006
307	19-01-11	SN	w, 10M-01	307_110119_SN10M-01w	JX213143
310	19-01-11	SN	w, U-01	310_110119_SNU-01__w	JX213144
312	19-01-11	SN	f (*Salmo salar*), 09M-02	312_110119_SN09M-02f	JX213007
314	01-02-11	SN	f (*Salmo salar*), 09M-01	314_110201_SN09M-01f	JX213009
320	01-02-11	SN	f (*Salmo salar*), 10M-09	320_110201_SN10M-09f	JX213011
326	01-02-11	SN	f (*Salmo salar*), UR-01	326_110201_SNUR-01_f	JX213012
330	01-02-11	SN	f (*Salmo salar*), UR-01	330_110201_SNUR-01_f	JX213016
333	01-02-11	SN	w, UR-01	333_110201_SNUR-01_w	ns
339	08-02-11	SN	w, U-02	339_110208_SNU-02__w	JX213149
348	08-02-11	SN	w, U-02	348_110208_SNU-02__w	JX213154
352	15-02-11	SN	f (*Salmo salar*), 09M-03	352_110215_SN09M-03f	JX213025
365	08-02-11	SN	f (*Salmo salar*), 09M-01	365_110208_SN09M-01f	JX213032
381	21-02-11	SN	w, 09M-03	381_110221_SN9M-03_w	JX213208
394	21-02-11	SN	w, U-03	394_110221_SNU-03__w	JX213171
402	14-03-11	SN	w, 09M-01	402_110314_SN09M-01w	ns
403	14-03-11	SN	w, 09M-01	403_110314_SN09M-01w	JX213176
404	14-03-11	SN	w, 09M-01	404_110314_SN09M-01w	ns
409	14-03-11	SN	w, U-02	409_110314_SNU-02__w	ns
411	28-03-11	SN	s, pre-treatment	411_110328_SNPretrts	JX213178
418	28-03-11	SN	w, pre-treatment	418_110328_SNPretrtw	ns
432	14-03-11	SN	f (*Salmo salar*), 06M-15	432_110314_SN06M-15f	JX213052
433	28-03-11	SN	s, post-treatment	433_110328_SNPostrts	JX213187
438	04-04-11	SN	w, U-02	438_110404_SNU-02__w	JX213189
449	04-04-11	SN	f (*Salmo salar*), 10M-02	449_110404_SN10M-02f	JX213059
489	10-04-11	SN	f (*Salmo salar*), 10M-01	489_110810_SN10M-01f	ns
492	29-08-11	SS	f (*Salmo salar*), anesthetic bath	492_110828_SSAnes__f	ns
502	29-08-11	SS	f (*Salmo salar*), tank R31	502_110829_SSTnkR31f	ns
507	05-07-11	SN	f (*Salmo salar*), U-03	507_110705_SNU-03__f	ns
517	28-11-11	SN	f (*Salmo salar*), U-01	517_111128_SNU-01__f	ns
554	27-02-12	SS	f (*Salmo salar*), tank R10	554_120227_SSTnkR10f	ns

naturation (30 s at 95°C), annealing (45 s at the optimal temperature determined for each primer), and extension (2 min at 72°C), and a final extension (7 min at 72°C). Post reaction samples were held at 4°C until processed. A 5.0 µl volume of each PCR product was mixed with 2 µl of 1:10 diluted loading dye (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 30% v/v glycerol in dH_2O) and loaded into an agarose gel. Amplification products were separated by electrophoresis on a 50-lane, 2.5% w/v agarose gel (150 V for 1 h and 36 min) that included a 100 bp DNA ladder (New England BioLabs) and visualized by staining with GelRed nucleic acid gel stain (Biotium) for 60 min (3× staining solution from 10 000× stock, w/v), followed by illumination under UV light, digital image capture using the GelDoc XR+ Gel Documentation System, and subsequent image analysis with Image Lab Software (Bio-Rad Laboratories Canada). Each primer and isolate combination was repeated at least 3 times with the same DNA extracted from the original pure culture isolate to ensure reproducibility and consistency in the final results.

Scoring of amplification profiles for genotypic analysis

Each gel image was scored independently and contained the amplification products of 46 isolates of *S. parasitica*. Minor image adjustments and lane selections were completed using Image Lab Software to optimize band visualization. Bands were manually selected and the sizes of amplified fragments were calculated using the 100 bp ladder standard and Analysis Toolbox functions of the Image Lab Software to determine the molecular weight (bp) and the absolute quantity (ng) of DNA in each amplicon

detected. The Analysis Table functions were used to compile and export the recorded data for each gel image to a Microsoft Excel spreadsheet, and the presence (1) or absence (0) of all bands at each locus detected was manually scored for each isolate recorded on a gel image. Three replicate amplification profiles for each unique isolate and primer combination were completed, compiled and aligned to create a single amplification profile that represented all bands amplified in at least 1 of the 3 replicates (example provided in Fig. 2). Bands from each replicate were aligned based on calculated base pair size, relative position to other bands, overall band pattern, and band intensity. The average base pair size data determined from the alignment of the 3 replicate gels was used to score the presence or absence of a given character (amplicon) for each isolate tested.

Population analysis

For population genetic analysis, all of the character trait scores (amplicon presence or absence, 1 or 0, respectively) for a given isolate and primer combination were ordered (in primer alphabetical order) into a single string of characters, for each isolate of *S. parasitica* included in this study. Phylogenetic trees were derived using distance matrix data from POPGENE (version 1.32) (Yeh et al. 1997), which also calculated the observed (N_a) and effective (N_e) number of alleles, number of polymorphic loci, genetic distance, Shannon's information index (I), and Nei's genetic distance (H_e), which is a measure of allele changes or codon substitutions at ≥1 gene loci, for any pair of taxa under consideration, in order to determine the extent of divergence over space and/or time. Characters were assumed to be dominant markers from a diploid data set in Hardy-Weinberg equilibrium, and the hierarchical structure was set to multiple populations. Maximum parsimony trees were created using Pars of PHYLIP (version 3.695) (http://evolution.gs.washington.edu/phylip.html). Parsimonious trees were made using default settings except for saving 1000 trees and randomizing input order (jumble = 10). Consense of PHYLIP was used to make a consensus tree of the parsimonious trees. A majority consensus tree of bootstrapped data was thus created using Seqboot, Pars, and Consense of PHYLIP. MEGA (Build# 4028) (Tamura et al. 2007) and FigTree (version 1.4.0) (Rambaut 2012) were used to display and label trees.

Isolates obtained from the Sayward North facility were subject to further analysis by conducting pairwise comparisons to detect significant temporal trends for genetic variability. The genetic distance (H_e) between pairs of isolates was plotted against the number of days between sample collections, and a regression analysis was completed to describe the relationship among isolates.

RESULTS

Composition of isolate collection

Samples were collected from a number of sources in British Columbia to test for the presence of *Saprolegnia* spp. (Table 1). When this study was initiated, the potential sources of inoculum were undetermined. Consequently, a number of sources were initially tested for *Saprolegnia* spp., including facility well water, water from pre- and post-filter systems, bio-filter components, and fish feed. Over the sampling period (2009 to 2012), the majority of the isolates used in phylogenetic analysis were obtained directly from fish tissue and water samples collected at different locations in aquaculture facilities, while a smaller number came from eggs and swabs of work surfaces. A total of 580 pure culture isolates were collected from aquaculture facilities, and the majority (350) were confirmed as *S. parasitica* by nucleotide sequence analysis of the ITS region. Among these 350 isolates, 177 were obtained from fish (51%), 152 from water samples (43%), 12 from swabs (3%), 8 from eggs (2%), and 1 from the bio-filter system (<1%). The occurrence of saprolegniosis in fish populations was a chronic problem, causing low levels of infection and mortality at all facilities during the study period. The majority of the samples were collected at different locations and times within the Sayward Hatchery North facility (Table 1) to conduct a temporal survey of *S. parasitica* isolates at a single location.

Primer development for population genetic analysis

Primer pairs tested for conventional microsatellite markers identified in the *S. parasitica* EST library (Torto-Alalibo et al. 2005) were not effective for evaluating genetic variability among isolates in the current project. Amplified products were not always reproducible and often had a limited number of amplicons for a given primer pair–isolate combination (data not shown). Each primer pair was specific to an EST sequence and the targeted SSR showed

low variability, with some longer repeat sequences occurring less frequently (Table 3). These SSRs were not useful for the assessment of intraspecific variability among potentially closely related genotypes, which prompted the evaluation of RAMS degenerate primers that amplify more common SSR sequences.

From the 350 confirmed isolates of *S. parasitica*, 87 were selected to evaluate their genetic variability through the use of RAMS genetic markers (Table 2). The isolates were divided into 2 sets of 46 isolates each, and isolates 104, 147, 179, 221, and 231 were shared between both sets to allow the valid comparison of fingerprint amplicons visualized on separate gels. For DNA extractions of this isolate collection, most samples had a DNA concentration of 200–500 ng µl^{-1} and a purity of 1.9–2.1 (based on $A_{260\ nm}$/$A_{280\ nm}$). Degenerate PCR primers and optimal annealing temperatures are summarized in Table 4 and include 7 primers that were complementary to different doublet or triplet SSRs in the genome. Two primers designed previously by Hantula et al. (1996) were used, in addition to 5 novel primers developed in the current study. Only those primers that provided reproducible results in our initial screening were used

Table 3. Summary of primer pairs developed from the expressed sequence tag (EST) library of *Saprolegnia parasitica* isolate ATCC 90214 (Torto-Alalibo et al. 2005) and evaluated in the current study. For each EST containing simple sequence repeats (SSRs), the primer sequences, estimated product size, primer start position, percent GC, melting point (T_m), repeat sequence detected and copy number, and percent match are indicated

EST name (acc. no.)	Primer pair sequence (5′–3′)	Product size (bp)	Primer start	% GC	T_m	Repeat sequence and copy number	% Match
SPM12F12(3)	F: AGCAGCAACAACAGATGCAC	235	351	50.0	60.1	$(CAG)_{9.7}$	100
(DN615994)	R: ATGTAACGGTTCTGCTAGGT		585	45.0	60.1		
SPM10F9	F: ATGCACGAGCTGCACAAG	159	22	55.6	59.7	$(AAG)_{11.3}$	100
(DN615782)	R: GCTATGCTAGTCGCTCGAG		180	57.9	59.7		
SPM17B4	F: ACGATGACGACGACCTCTCT	152	118	55.0	59.9	$(CGA)_{8.7}$	100
(DN615844)	R: GCAAACCTGTTATTGGGCTG		269	50.0	60.2		
SPM6C12	F: GAGACGACCTCGATGACCA	229	69	57.9	59.8	$(CGACGC)_{4.2}$	100
(DN616516)	R: GCTCTGCATGGACGGTTTCA		297	55.0	60.3		
SPM9E2	F: GAGACGACCTCGATGACCA	229	76	57.9	59.8	$(CGACGC)_{4.2}$	100
(DN616134)	R: GCTCTGCATGGACGGTTTCA		304	55.0	60.3		
SPM12F12(9)	F: AGCAGCAACAACAGATGCAC	235	351	50.0	60.1	$(GCAGCAGAT)_{10.6}$	83
(DN615994)	R: ATGTAACGGTTCTGCTAGGT		585	45.0	60.1		
SPM22H4	F: GTTTAATTGCTTTTTGTGGTTTAAT	368	57	24.0	57.2	$(TTAAAAATT)_{6.2}$	80
(DN616963)	R: ATACGGAGTTAAGCTAGTCAAAA		424	34.8	59.9		
SPM5A11	F: CCAGCGAGAATCGAAGAATG	246	25	50.0	60.9	$(GGCAAGGGC)_{5.1}$	91
(DN616361)	R: TAGTACCTCATGGAGTGCCG		270	55.0	59.9		
SPM24A11	F: CCTCCAGGCATTCTACGAAA	215	213	50.0	60.2	$(GACGATGAT)_{3.6}$	95
(DN616950)	R: ATGTGGACTTCTCTTGGGCG		427	55.0	60.3		
AJ413215	F: GAAGACAGCGTGTACGTGGA	228	1040	55.0	59.9	$(CATCACCAC)_{4.9}$	82
(AJ413215)	R: TTGACAGGTTCCGTTGTAGG		1267	50.0	59.9		
SPM14G12	F: GTGCGTTTGCCGAGTTTCT	249	419	52.6	61.4	$(TCTTCCTCC)_{3.3}$	95
(DN616839)	R: TATTGCTGCTGCTGCTGAGG		667	55.0	60.1		
SPM3G11(9)	F: GAGGGGTCAACAACGATCC	229	283	57.9	60.3	$(CGACGATCT)_{5.7}$	77
(DN616111)	R: TACCAGCTCCGAATGCAGTG		511	55.0	60.1		
SPM9C7	F: CGTCTACGTGGATGCAAAAG	250	62	50.0	59.3	$(GACAGCAGC)_{4.7}$	100
(DN617197)	R: AGAAGCCAGAGCCAGAGTAG		311	55.0	60.4		
SPM8G10	F: CCGGTCTGGACGTGGTTT	247	38	61.1	61.9	$(CGGTGGTCGTGG)_{4.5}$	79
(DN617061)	R: CGTTGTTCCAGTAGCAGCTC		284	55.0	61.8		
SPM4H7	F: ACGGCAAGGTCGAAGTCAT	208	328	52.6	60.7	$(CGACGCCCAAGC)_{3.9}$	85
(DN616629)	R: CTCTTCACGCCACTGACGTA		535	55.0	61.4		
SPM3G11(12)	F: GAGGGGTCAACAACGATCC	229	283	57.9	60.3	$(CGATCTGACGA)_{6.1}$	78
(DN616111)	R: TACCAGCTCCGAATGCAGTG		511	55.0	60.1		
SPM4C11	F: CCACGCGTCCGTTAAAAT	180	6	50.0	59.5	$(AAAAAAAAATCA)_{2.7}$	100
(DN616577)	R: TTCCTGGCAATCCAAGTTAACT		185	40.9	59.9		
SPM5F9	F: ATGAAGCTGGCAGAGAGCAT	210	151	50.0	60.1	$(GACGTGGACATG)_{2.4}$	100
(DN616344)	R: GGTTTCGGAAAGTCTGTTGT		360	45.0	58.9		

Table 4. Degenerate 5′-anchored primers used for genetic marker analysis included primers developed by Hantula et al. (1996), as well as new primers developed in the current study. Nucleotide ambiguity code (IUPAC): V (A, C, G), H (A, C, T), D (A, G, T), B (C, G, T), N (any base). Indicated for each primer are the percent GC content, melting point temperature (T_m), the temperature gradient tested to determine the optimal annealing temperature for PCR amplification, and the optimal annealing temperature identified during the screening of each primer

Primer name	SSR sequence	Primer sequence (5′–3′)[a]	GC content (%)	T_m (°C)	Temp. gradient (°C)	Optimal annealing temp. (°C)
GT[a]	GT	VHVGTGTGTGTGTGN	54.4	53.1	58 ± 5	55
ACA[a]	ACA	BDBACAACAACAACAACA	37.0	47.8	49 ± 10	48
CCA[b]	CA	DDCCACCACCACCACCA	62.7	58.1	61 ± 10	64
BCAG[b]	CAG	BDBCAGCAGCAGCAGCAG	64.8	59.5	64 ± 5	61
DAAG[b]	AAG	DDAAGAAGAAGAAGAAG	33.3	40.9	50 ± 10	42
DAGG[b]	AGG	DDAGGAGGAGGAGGAGG	62.7	53.3	64 ± 5	61
DAGC[b]	AGC	DDAGCAGCAGCAGCAGC	62.7	57.6	64 ± 5	61

[a]Primers used previously by Hantula et al. (1996); [b]Primers developed in the current study

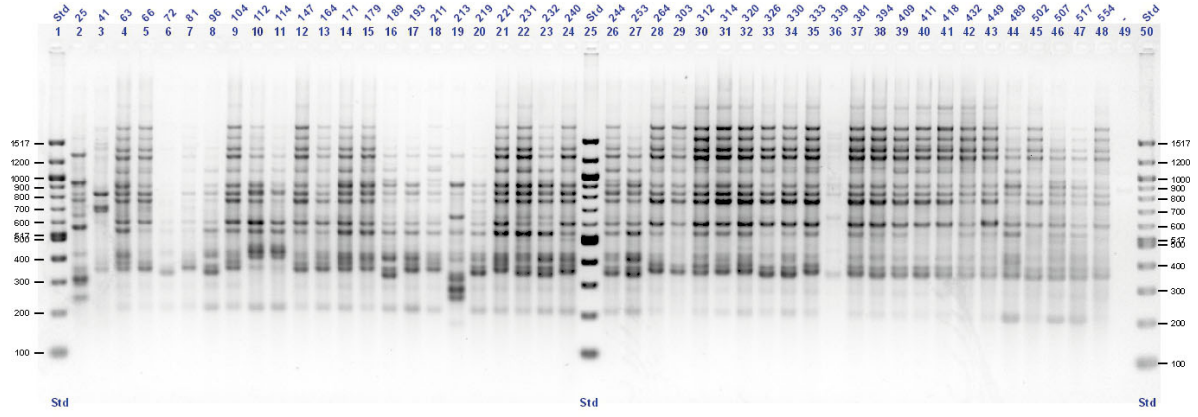

Fig. 2. Amplification of random amplified microsatellite (RAMS) markers using the degenerate primer 5′-BDB(ACA)$_5$-3′. See 'Materials and methods: Scoring of amplification profiles for genotypic analysis' for details of gel scoring and analysis. Isolate number and amplified DNA size standard (Std, 100 bp ladder) are shown

for the analysis of all selected isolates. The number of amplicons generated by a given primer ranged from 23 to 44, with fragment sizes ranging from 145 to 3000 bp. This variation in fragment sizes is due to length variation in the amplified sequences between primer sites, as well as the occurrence of single nucleotide polymorphisms (SNPs) or deletions that added or eliminated primer annealing sites. An example of results is provided in Fig. 2, showing fingerprints for selected *S. parasitica* isolates that were amplified using the degenerate RAMS primer ACA (Table 4).

Population analysis

For the collection of 87 *S. parasitica* isolates amplified with 7 RAMS primers, a total of 309 characters (amplicons) were scored, providing data for population genetic analysis. A total of 96% (297) of the loci were polymorphic. For the purpose of this study, each amplicon is considered as a dominant marker that will show Mendelian inheritance in progeny. This was demonstrated by Zietkiewicz et al. (1994) in a 3-generation human pedigree study that examined the amplicons produced by single RAMS primers. They considered some polymorphic bands (of a similar size) to be allelic length variants of the same locus or amplified region, since closely related individuals in their study shared many amplicons of similar length, while the more distantly related individuals shared fewer amplicons. In the studies of Hamberg et al. (2017, 2018), RAMS amplicon sequences were used to evaluate Mendelian inheritance in the basidiospore progeny obtained from crosses between Canadian and Finnish genotypes of *Chondrostereum purpureum*, a wood-decomposing basidiomycete fungus. These genetic markers provided consistent sizes of amplicons over 2 generations and demonstrated the independent assortment of these reliable markers among basidiospore progeny.

Table 5. Summary statistics for *Saprolegnia parasitica* population genetic analyses. Data are means ±SE where applicable

Variable	Value
Number of isolates	87
Total number of characters	309 (from 23 to 44 for each primer)
Number of polymorphic loci (%)	297 (96%)
Observed number of alleles (N_a)	1.96 ± 0.19
Effective number of alleles (N_e)	1.36 ± 0.33
Nei's genetic distance (H_e)	0.23 ± 0.17
Shannon's information index (I)	0.36 ± 0.22

We determined the observed number of alleles (N_a) for the population to be 1.96 ± 0.19 (mean ±SE), while the average effective number of alleles for all loci (N_e) was 1.36 ± 0.33. The effective number of alleles is a measure of true diversity within the population (Jost 2008). Nei's genetic distance (H_e) was 0.23 ± 0.17 (Nei 1987) and Shannon's information index I (Lewontin 1972) was 0.36 ± 0.22, both indicating a low level of genotypic diversity in this population (Table 5).

A majority consensus tree was constructed (Fig. 3) to determine the relatedness of collected isolates (e.g. isolates associated with specific locations). Most bootstrap values were low, but those greater than 0.60 are indicated and noted with a unique clade identifier. Of particular interest is clade A (0.83), which is composed of a geographical mixture of isolates including nos. 25 and 30 (PR; site codes are given in Table 1), 34 (UG), 41 and 42 (NR), and 213 (SN). Each of the pairs of isolates nos. 411 and 418 (SN, 0.90, clade F), 432 and 449 (SN, 0.69, clade E), 72 and 81 (UH and SN, respectively, 0.69, clade D), 112 and 114 (GL, 0.99, clade C), and 221 and 231 (SN, 0.91, clade B) clustered together and, in each case, were assumed to be similar genotypes, based on this analysis and the presence of many shared amplicons. No correlation was observed between sample substrate type and the genotype of isolates tested in this analysis.

In order to explore the relationship between collection date and genetic distance, a scatter plot of genetic distance between pairs of isolates versus the number of days between sample collection at Sayward Hatchery North was generated (Fig. 4); this facility was sampled more intensively over an extended period of time. This analysis allowed us to visualize the range of genetic distances among isolates over time. Due to the low number of sample pairs with greater than 210 d between sample collections, the analysis only included sample collections with up to 210 days of difference. Over this time range, there appeared to be a clustering of values with a genetic distance (H_e) between 0.1 and 0.3. The variability in genetic distance among samples decreased as the number of days between their collections increased; there was higher variability in genetic distance observed among samples collected closer together. However, no significant relationship was detected between genetic distance among isolates and the number of days between isolate collection at this site. The linear equation could not explain most of the observed variability in genetic distance among isolates.

DISCUSSION

This study conducted in British Columbia, Canada, represents one of the first large-scale surveys to examine the distribution of *Saprolegnia parasitica* genotypes over a range of geographical sample locations. A total of 350 pure culture isolates were obtained from a range of source material and their species identity confirmed by ITS-rDNA region sequence analysis and comparisons to confirmed species isolates of the genus *Saprolegnia*. A subset of this collection (87 isolates) was used for further population analysis to evaluate population genetic variability in relation to substrate origin, sample location, and temporal sampling regimes.

Among the range of sample substrate types (water, fish tissue, eggs, and surface swabs), our phylogenetic analysis did not identify a correlation between substrate type and specific *S. parasitica* genotypes. No correlation was detected between species genotype and isolates obtained from infected host tissue or eggs, which suggests that all genotypes possess a similar level of pathogenicity, and that conditions in the aquaculture environment have not selected for a limited group of more effective, fish-pathogenic *S. parasitica* genotypes. These findings support the opportunistic nature of this pathogen, which is ubiquitous in freshwater systems and may be sustained on both living hosts and a range of non-living organic substrates (Bruno et al. 2011). This plasticity of *S. parasitica* in terms of its nutritional substrate preference can make its management in aquaculture facilities challenging, as once the pathogen is introduced, it may be very difficult to remove completely from the system.

The commercial aquaculture facilities included in this study may share isolates of *S. parasitica* as a consequence of the transfer of eggs and juvenile salmon

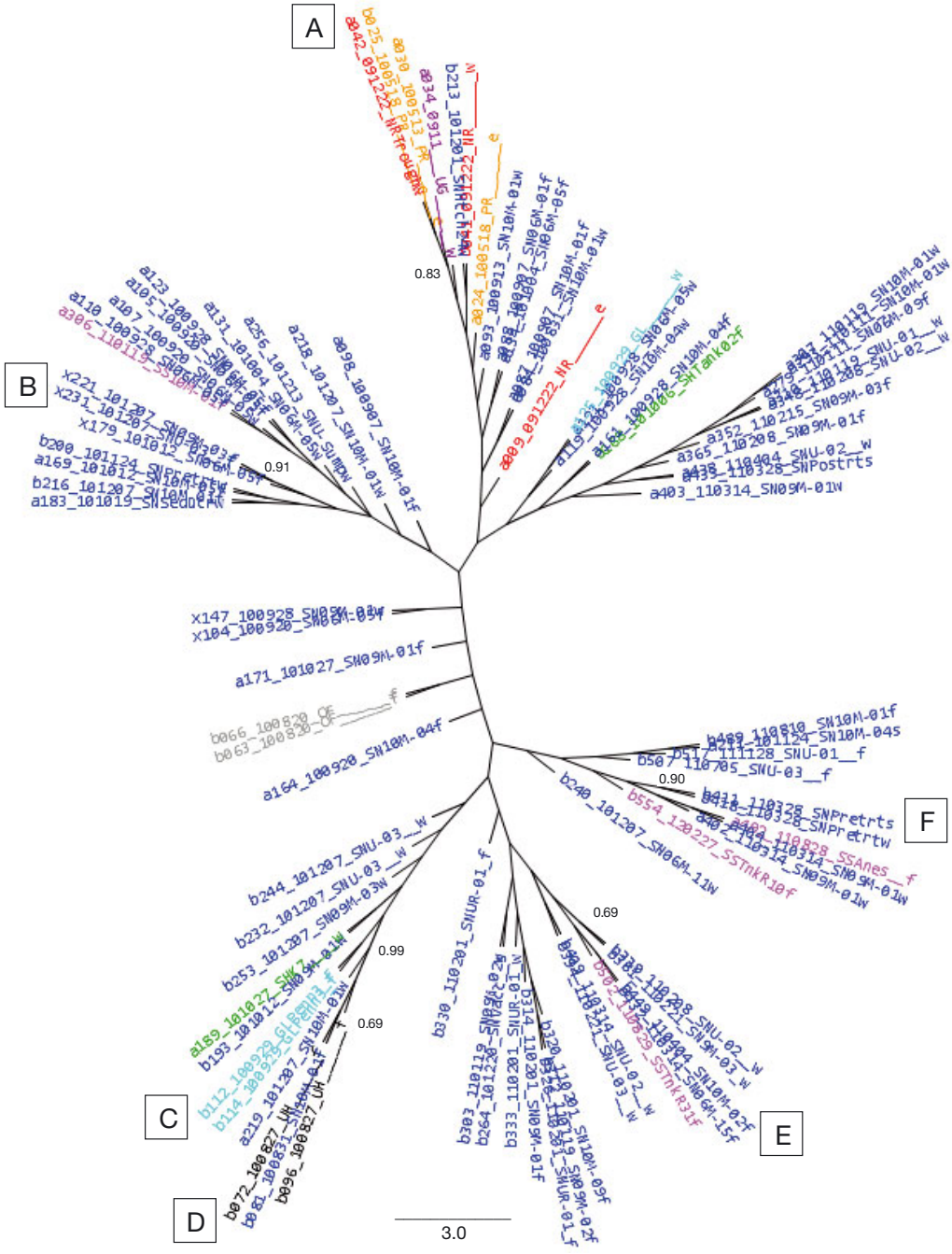

Fig. 3. Majority consensus of maximum parsimony analysis of bootstrapped data for all isolates. Isolates are color-coded according to sample collection location: Sayward North (blue), Nanaimo River (red), Stelling Hatchery (green), Georgie Lake (aqua), Puntledge River (orange), Upper Goldstream (purple), Sayward South (pink), Ocean Falls (grey), and United Hatchery (black). Bootstrap values greater than 0.60 are indicated, and specific clades are identified by letter

among facilities, which typically occurs on a semi-annual basis (B. Boyce pers. comm.). For example, United Hatchery (UH) supplies fish eggs and juvenile fish to other facilities, such as Sayward Hatchery North (SN); infected fish could therefore provide a source of *S. parasitica* inoculum for other facilities. These facilities also receive eggs or juvenile salmon from other locations outside of those sampled in this study. Due to the potential for regular exchange in genotypes among facilities, we considered isolates obtained from these different locations to belong to a single *S. parasitica* population. The variability of

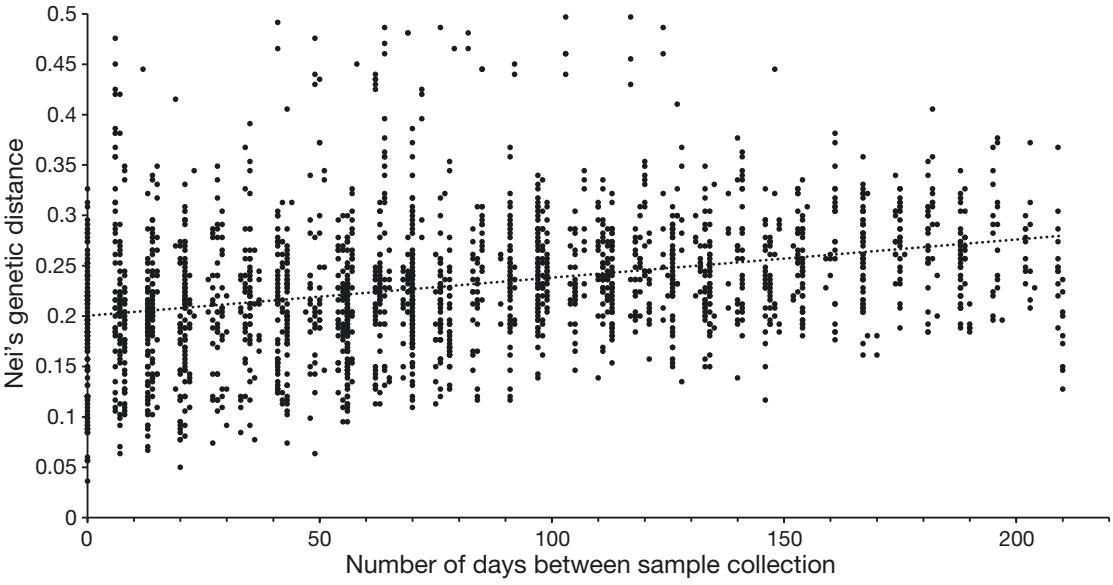

Fig. 4. Relationship between Nei's genetic distance and sampling interval for *Saprolegnia parasitica* isolates collected at Sayward Hatchery North between 31 August 2010 and 28 November 2011. Patterns of temporal genetic variation in this population were described by calculating Nei's genetic distance, making pairwise comparisons of isolates collected at different time intervals (number of days apart) to determine their genetic similarity or divergence over time. Each value shown is an estimate of the genetic difference between 2 individuals in this population, as determined by a comparison of the data compiled for all RAMS loci assessed in this study. Best fit equation $y = 0.0004x + 0.2004$ ($R^2 = 0.0921$). No significant relationship was detected between genetic distance and the number of days between isolate collection. Pairs of isolates greater than 210 d apart were not included due to low sample sizes

genotypes observed among facilities will have their origins in both natural sources of inoculum (e.g. local water sources), and the accidental introduction of genotypes on colonized fish, eggs, and in the associated water from other locations. One other consequence of this genotypic diversity may be an increased likelihood for sexual events and the generation of new genotypes in these artificial systems.

The genetic variability of selected *S. parasitica* isolates was described by a genome-wide assessment of SSR microsatellite-associated markers. Single degenerate primers annealed to the 5′ end of doublet or triplet repeats to provide a range of amplicons for each primer–genotype combination. Reliable primers that provided reproducible amplification profiles were used to detect a total of 309 characters, 96% of which were polymorphic among the 7 primers used in the assessment of this population. Polymorphisms were likely due to a number of scenarios, including the presence of SNPs that alter primer binding sites, insertions and deletions that change amplicon length, or insertions between primer binding sites that create sequences too long for effective PCR amplification (Zhivotovsky 1999, Altukhov 2006). The effective number of alleles was close to the minimum value of 1 (1.36 ± 0.33), indicating that there was a very uneven distribution of alleles, with single alleles dominant and a low frequency of rare alleles in the population. It was evident that many amplicons were shared among the selected isolates and the relatively high polymorphism we observed (96%) was due to a small number of isolates that were more variable, when compared to the remainder of the population. Overall, the genetic diversity of this population was determined to be relatively low, based on the indices used for this analysis (Table 5).

Parsimony analysis did not detect specific clades of genotypes correlated with the 9 different sample locations of the *S. parasitica* isolates, as demonstrated in the mixed composition of isolates found among clades A to F (Fig. 3). The low Shannon's information index ($I = 0.36 \pm 0.22$), compared to the high percentage of polymorphic loci (96%) supports our observation that a small number of isolates are creating most of the diversity within the isolate collection. For example, isolates clustering in Clade A (25, 30, 34, 41, 42, and 213) represented a distinct group compared to the rest of the isolates. The isolates that make up clade A were collected between November 2009 and May 2010 at 4 different geographical locations (Puntledge River, Nanaimo River, Sayward North, and Upper Goldstream). Samples from the Upper Goldstream site were collected from a body of water outside of an aquaculture facility. Despite their disparate

origins, these isolates may have clustered together because the majority of *S. parasitica* isolates analyzed in this study are more similar to each other than to the isolates in clade A. Other clades noted in this analysis, specifically clade B (SN, isolates 221 and 231), clade C (GL, isolates 112 and 114), clade E (SN, isolates 432 and 449), and clade F (SN, isolates 411 and 418), included isolates from the same location and were likely very similar genotypes.

Most of the clades with significant bootstrap values contained isolates collected within a short time frame from the same location. These isolates may well represent clonal genotypes of the same individual, or closely related progeny of sexual events. We did observe genotypic variation over time at Sayward Hatchery North in the form of different clades of isolates (e.g. SN, clades B and F) and it is possible that sexual recombination is generating a certain amount of diversity. In addition, there may have been introductions of novel genotypes during this same time period; this was supported by the similarity between isolates in clade D (SN isolate 81 and UH isolate 72), where fish transfers from UH to SN may have introduced a new genotype. It is difficult to distinguish the relative contribution of each source (introductions and recombination events) to genetic variability, but it is clear that we did not observe a single persistent clonal population of *S. parasitica* in SN over the sampling interval.

The temporal study (Fig. 4) evaluated the relationship between genetic distance (H_e) and the number of days between individual sample collections over a 15 mo period in 1 facility. The population sampled did demonstrate some genetic variability, but there was no significant change in diversity or a clear linear trend, when considering the number of days between *S. parasitica* sample collections at this location. This suggests that significantly novel genotypes were not being introduced into the facility during the study interval from either fish transfers or local water sources. A continuous population of *S. parasitica* effectively exists among these Atlantic salmon aquaculture facilities through the movement of water and fish stocks at regular intervals, activities that effectively allow gene flow among these seemingly isolated artificial freshwater systems. The extent of transfers via fish stocks is difficult to quantify, but we have observed that apparently healthy fish (asymptomatic for saprolegniosis) can still yield viable cultures of *S. parasitica*, which undermines the efforts of fish health mangers to contain unhealthy fish stock. In addition, within individual facilities, multiple clonal lineages of *S. parasitica* may undergo rare sexual recombination events that generate variability over time, thus contributing to some of the observed variability in this temporal study. In summary, the temporal study does demonstrate that, in this aquaculture system, there does not appear to be the selection of more pathogenic *S. parasitica* genotypes associated only with a fish host, or the persistence of a single clonal genotype that causes recurring outbreaks of saprolegniosis. Overall, the management of this disease in freshwater facilities will need to consider the presence of established populations of *S. parasitica*, the unintended introduction of the pathogen with fish and egg transfers, as well as routine measures to minimize stressors that may increase host susceptibility to infection at different life stages. Both water quality and fish health monitoring, along with targeted water treatments, will be required until more effective anti-oomycetous agents or vaccines are developed.

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