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Growth and sporulation differences of genetically distinct *Saprolegnia parasitica* strains *in vitro*

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ABSTRACT: *Saprolegnia* species are major oomycete pathogens of freshwater fishes, causing significant economic and ecological losses in aquaculture and wild populations. Most saprolegniosis disease outbreaks have been attributed to single or very few genetic clones, but potential differences among strains and the covariation of their virulence and transmission traits have remained largely unresolved. In this study, we investigated differences in laboratory growth and sporulation among 9 pathogenic *S. parasitica* isolates originating from infected salmonids from 6 aquaculture facilities and 1 wild waterbody across Finland. All isolates were assigned to distinct strains using either specific qPCR or by sequencing the internal transcribed spacer, species identification was confirmed and unified by genome reassembly, and phylogenetic analysis was performed to characterise genetic relationships among the strains. Hyphal growth, time to initiate spore production, spore numbers, and count of repeated zoospore emergence cycles were measured, and potential phenotypic associations among these functional measures were assessed. The strains formed 3 distinct clades with 7 out of the 9 strains clustering into clade I, whereas clades II and III were singletons. Phylogenetic clade, timepoint, and their interaction all significantly affected hyphal growth, indicating timepoint-dependent clade differences. Strains within clade I were faster to initiate sporulation and produced more spores than the singleton clades II and III. Associations between hyphal growth and sporulation metrics did not support trade-offs between transmission- and virulence-related traits; strains with fastest growth and sporulation produced the most spores.

KEY WORDS: *Saprolegnia parasitica* · Intraspecific variation · Hyphal growth · Sporulation · Phylogenetic analysis · Aquaculture pathogens

1. INTRODUCTION

Anthropogenic environmental changes can significantly influence the ecology and evolution of opportunistic pathogens (Mennerat et al. 2010, Berngruber et al. 2013). Rapid virulence evolution, especially in high-density environments such as animal farms and aquaculture facilities, may contribute to the emergence of many human and animal diseases and, at worst, global pandemics (André & Hochberg 2005, Kennedy et al. 2016). In crowded systems, elevated host contact rates and continuous host availability weaken the trade-off constraints that ordinarily limit pathogen exploitation (André & Hochberg 2005, Alizon et al. 2009), favouring variants that grow faster, transmit more efficiently, or cause more severe disease (Mennerat et al. 2010, Kennedy et al. 2016). This selective landscape not only accelerates the emergence of high-virulence pathogen genotypes but can also promote the spread of certain clonal lineages, shaped by recurrent transmission bottlenecks, host homogeneity, and feedback between infection dynamics and intensive management practices (Mennerat et al. 2010, Pulkkinen et al. 2010, Kennedy et al. 2016).

The genus *Saprolegnia* includes over 30 filamentous aquatic oomycete species, commonly known as 'water moulds' (Sandoval-Sierra et al. 2014, Pavić et al. 2022), many of which are opportunistic pathogens causing

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saprolegniosis, a disease characterised by cotton-like hyphal growth on the skin, gills, fins, and fish eggs (van West 2006). These species exhibit saprotrophic and necrotrophic qualities outside live hosts but infect fish and amphibians especially when these are under stress (Pavić et al. 2021, Eszterbauer et al. 2025). The highly pathogenic *S. parasitica* (Coker, 1923) is the most economically and ecologically significant species, responsible for large-scale disease outbreaks in both aquaculture and wild fish populations (van den Berg et al. 2013, Sandoval-Sierra et al. 2014, Ravasi et al. 2018). Fishes suffering from saprolegniosis include not only salmonids but carps, catfish, sturgeons, and Eurasian perch. While saprolegniosis is relatively rare among wild fish, except for spawning salmonids (Rocchi et al. 2017, Härkönen et al. 2024), it can spread as an epidemic, often as a coinfection with other pathogens, resulting in mass mortalities even within just a few days of first visual detection of the disease (Hatai & Hoshiai 1992, Moharam et al. 2025). There are no known cures for the disease, and prophylactic measures can prevent outbreaks only to a limited extent (Benavent-Celma et al. 2022). Although many hatchery outbreaks are dominated by at most a few clones (Diéguez-Uribeondo et al. 2007, Ravasi et al. 2018, Engblom et al. 2023), the extent of biologically relevant intraspecific variation in *S. parasitica* has remained unclear. However, for the management of the disease, it would be crucial to know if *S. parasitica* isolates show functional differences indicative of microevolutionary differences reflecting adaptation to certain host species or high-density host environments (André & Hochberg 2005, Pulkkinen et al. 2010, Borovkov et al. 2013).

S. parasitica show qualities that make them potentially highly evolvable and genetically diverse (Jiang et al. 2013), e.g. *Saprolegnia* spp. may reproduce sexually in certain challenging conditions (Ali et al. 2013, van den Berg et al. 2013). Certain oomycetes (Chamnanpunt et al. 2001, Lamour et al. 2012), including *S. parasitica*, show significant mitotic loss of heterozygosity which suggests the capacity to rapidly develop genetically diverse clones (Jiang et al. 2013). A few studies have also demonstrated variation in pathogenicity (Stueland et al. 2005, Thoen et al. 2011), in addition to physiological characteristics and host preference among and within different *Saprolegnia* species (Matthews et al. 2021, Pavić et al. 2022, Erdei et al. 2023). However, it has remained somewhat unclear whether and how genetic relationships of *S. parasitica* isolates are reflected in the variation in how they grow in standard laboratory medium. Furthermore, potential correlations or trade-offs between functional traits have not been addressed in the context of transmission- and virulence-related traits.

In Finland, *S. parasitica* causes significant losses in salmonid aquaculture, particularly affecting *Salmo salar*, *Salmo trutta*, and *Oncorhynchus mykiss* (Janhunen et al. 2019). Anecdotal evidence suggests that saprolegniosis epidemics vary in severity within, as well as across facilities, pointing to potential intraspecific differences beyond site-specific factors. This is further supported by studies based on a few genetic markers showing that within Finland, *S. parasitica* comprises several genetically different types (Bangyeekhun et al. 2003, Engblom et al. 2023). Theoretically, hyphal growth serves as an indicator of virulence through rapid host tissue colonisation leading to host death (van West 2006, Bruno et al. 2011). Repeated zoospore emergence, proposed to be an adaptation to parasitic life histories in *Aphanomyces* spp. (Cerenius & Söderhäll 1985) and demonstrated in certain *S. parasitica* lineages (Bangyeekhun et al. 2003), together with sporulation efficiency, represents transmission potential since spores act as infectious propagules that are carried by water (Walker & van West 2007). In high-density aquaculture environments, selection may favour high virulence over transmission due to abundant hosts, whereas transmission could be favoured in natural waters (André & Hochberg 2005, Alizon et al. 2009).

In this study, our aim was to examine (1) whether phenotypic variation in potential virulence- and transmission-related traits, via hyphal growth and sporulation efficiency as proxies, reflects phylogenetic relationships between *S. parasitica* strains, and (2) if covariation exists between these functional traits. Based on the theory of virulence evolution where virulence is strongly shaped by competition among pathogen strains within hosts (Alizon et al. 2009), we hypothesised that distinct phylogenetic groups would show high amounts of variation in the virulence-related functional traits. This approach can reveal functional diversity, with direct implications for understanding the role of strain variation in the development of *Saprolegnia*-induced mortalities in aquaculture conditions.

2. MATERIALS AND METHODS

2.1. *Saprolegnia parasitica* isolates

Saprolegnia parasitica cultures were isolated from 9 saprolegniosis-diseased salmonids originating from 6 anonymous aquaculture facilities and 1 natural water-

body located in different geographical regions of Finland (Table 1) (Engblom et al. 2023). All isolates were extracted from the surface of the skin of *S. parasitica*-infected host fish, rinsed with 70% ethanol and sterile water and placed on peptone-glucose (PG-1) agar (Unestam 1965) (Table A1 in the Appendix). Monocultures obtained from single spore cultures of each isolation were kept viable at 4°C on PG-1 agar covered with sterile paraffin oil. The cultures were renewed once a year by placing a small piece of mycelium onto new PG-1 agar with a sterile scalpel. The isolation renewal protocol was consistent across all isolates, i.e. all isolates were renewed simultaneously each year. All isolates represented distinct genetic strains: 3 isolates were assigned to strains VH102, VH162, and VH194 with specific quantitative PCR (Korkea-aho et al. 2022) and 6 isolates to strains VH10, VH112, VH28, VH62, VH70, and VH171 by sequencing the internal transcribed spacer region (Engblom et al. 2023) (Table 1). The species identification was confirmed and unified by genome reassembly. Five out of the 9 *S. parasitica* strains had been previously characterised with multilocus sequence typing (MLST) (Table 1) (Engblom et al. 2023).

2.2. Phylogenetic analysis

To investigate the genetic relationships between the strains, a phylogenetic tree was constructed based on 7 housekeeping genes previously used for *S. parasitica*-specific MLST analysis (ALTS1, COX1, GLUT, NAD1, RPB2, SHMT, TUBB) (Ravasi et al. 2018, Engblom et al. 2023). Here, we utilised Illumina whole genome resequencing reads (Q30 ≥ 90.36%) that were filtered and trimmed (0.05 error probability limit, >75 bp post trim length), and mapped to the *S. parasitica* reference genome strain CBS 223.65 (GenBank assembly accession no. GCA_000151545.2) in Geneious Prime v2026.0.2, using the native Geneious mapper with medium sensitivity and 5 iterations. Sequences of the 7 loci for all 9 strains are available in GenBank (Table A2). Multiple alignments of the 7 loci for the 9 strains and the reference strain were constructed using MUSCLE (Edgar 2004) and the alignments were concatenated using the native Geneious Prime tool. The concatenated alignments were used to construct a maximum likelihood phylogenetic tree based on the Tamura-Nei model of genetic distance (Tamura & Nei 1993), using PhyML version 3.3.20180621 (Guindon et al. 2010) with 1000 bootstrap replications.

2.3. Measurement of hyphal growth

A small section of each *S. parasitica* culture was sterilely transferred to a 100 mm Petri dish containing PG-1 agar (Unestam 1965), and incubated at room temperature (20°C) for 72 h. Subsequently, a 5.1 mm diameter plastic Pasteur pipette was used to excise an agar block colonised by *S. parasitica* hyphae from the advancing edge of hyphal growth. This was placed in the centre of a new 100 mm Petri dish and incubated under the same conditions, during which radial growth was measured (to 0.1 mm) every 24 h for a total of 72 h using a digital calliper; all measurements were made by a single person without any information about the phylogenetic relationships of the strains. For each measurement, the radial growth was determined from the edge of the 5.1 mm diameter agar plug to the furthest advancing point of the circular hyphal growth. Each strain was replicated on 5 agar plates (total n = 45) arranged in a randomised complete block design. Plates were placed on a laboratory bench in 5 rows (blocks), each row containing exactly 1 replicate of each strain (9 plates per row). Plate posi-

Table 1. *Saprolegnia parasitica* strains used in the study. ST: sequence type based on multilocus sequence typing (Engblom et al. 2023); nd: not determined

Strain	Fish species	Life stage	Date of sampling	Aquaculture facility ID	ST (GenBank accession no.)
VH10	*Salmo salar* m. *sebago*	Adult	10 Oct 2017	IS2	3
VH102	*Salmo salar* m. *sebago*	Juvenile	13 Dec 2018	PS12	nd
VH112	*Salmo salar*	Juvenile	2018	PS6	2
VH162	*Salmo trutta*	Adult	10 Oct 2019	PS12	nd
VH171	*Salmo salar*	Juvenile	11 Oct 2018	PS7	nd
VH194	*Salmo trutta*	Adult	3 Oct 2023	PS13	nd
VH28	*Salmo trutta*	Adult	26 Oct 2016	Wild	1 (OP629478.1)
VH62	*Oncorhynchus mykiss*	Juvenile	21 Jun 2018	IS7	2 (OP629503.1)
VH70	*Oncorhynchus mykiss*	Fry	5 Jul 2018	IS2	7 (OP679802.1)

tion was randomly assigned within each row to prevent positional bias. Consequently, no row contained more than 1 replicate of the same strain. The total area (mm^2) of the circular hyphal growth was determined based on the radius (r) as πr^2 at 3 time points.

2.4. Determination of sporulation rate

Using a 5.1 mm diameter plastic Pasteur pipette, an agar block colonised by *S. parasitica* hyphae was cut from the advancing edge of a hyphal growth and placed on a sterile Petri dish into a 300 µl droplet of PG-1 solution. Three replicates were made from each strain. Petri dishes were closed with a lid, sealed using parafilm, and incubated at room temperature (20°C) for 24 h. To initiate the production of spores, the hyphae were washed with sterile lake water 3 times at 1 h intervals by removing the water from around the hyphae using a Pasteur pipette and pipetting a new 300 µl droplet onto them. The lake water was collected from Lake Pyhäselkä, near Joensuu, Finland, from a single location at a single time point and used as 1 batch across experiments and replicates to ensure consistent water chemistry. The lake water was sterilised by autoclaving at 121°C for 2 h under saturated steam (200 kPa absolute pressure). The sterile lake water was stored at 4°C and used within 48 h after autoclaving. The production of sporangium and primary zoospores within the hyphae was investigated under a light microscope every 2 h for a total of 34 h (with an overnight 8 h break without observation after the first 12 h).

To count the number of spores produced, an agar block was cut as described in Section 2.3, placed into a sterile 50 ml Falcon tube with 45 ml of PG-1 solution and incubated at room temperature (20°C) for 48 h for further hyphal growth. Three replicates were prepared for each strain, along with a negative control containing PG-1 solution to monitor cross contamination. Replicates and the control were arranged on a laboratory bench using the same randomised layout as described for the agar plates. After the incubation period, the hyphae were poured onto sterile filter fabric to remove the PG-1 solution, then transferred back into the Falcon tube using a sterile loop, and 45 ml of sterile autoclaved lake water was added to each tube. The spore production was subsequently monitored by examining a small hyphal fragment on a microscope slide under a Zeiss Axioskop 2 Plus light microscope at 10 to 100× magnification, adjusting magnification as needed to observe hyphal growth and spore formation. When the first release of zoospores occurred, a subsample of 15 ml was taken from each of the 3 replicate Falcon tubes and placed into a new, sterile tube. Prior to sampling, each Falcon tube was inverted several times to ensure homogenous distribution of hyphae and spores. From the 15 ml subsample, 25 µl was pipetted into a Bürker chamber and the numbers of spores and zoospores in a 3-by-3 grid of large squares were counted. Spore counting was repeated 4 times for each tube. The concentration of spores ml^{-1} was calculated by dividing the total number of spores in the grid by 9 and then multiplying the value by 10 000 to convert the volume of a single large square (0.1 mm^3) to 1 ml. If motile zoospores were observed during the Bürker chamber counting, the Falcon tube containing the 15 ml subsample was vortexed for 45 s, and the number of spores and the presence of motile zoospores were determined after 150 min (for detailed method, see Diéguez-Uribeondo et al. 1994). The repeated zoospore emergence test (i.e. vortexing and waiting) was continued until no motile zoospores were observed. All microscopy and counting was performed by a single person without any information about the phylogenetic relationships of the strains.

2.5. Statistical analyses

The isolates were originally acquired for laboratory cultivation at different times (Table 1), which could have influenced the growth and sporulation of the strains over time (Songe et al. 2014). Therefore, we first applied linear regression models and Type II Wald χ^2 tests to determine whether the number of days each isolate had been maintained under laboratory conditions predicted variation in the strain-level mean hyphal growth (mm^2) at 48 h, time (h) to initiate spore production, number of spores produced (ml^{-1}), and number of repeated zoospore emergence cycles.

Differences in hyphal growth between phylogenetic clades and among individual strains were analysed using a generalised linear mixed-effect model (GLMM) with restricted maximum likelihood (REML) estimation. Here, hyphal area (mm^2) measured at the time points 24, 48, and 72 h was used as a response variable, with phylogenetic clade, timepoint, and their interaction included as fixed effects. Repeated measurements within each subject (strain × replicate) were modelled with a first-order autoregressive correlation structure to account for the strain-specific temporal dependence in growth trajectories. The residual distribution was specified as Tweedie (μ, ϕ, p) to accommodate the right-skewed and heteroscedastic growth data.

Differences in sporulation metrics between phylogenetic clades and among individual strains were analysed using GLMMs with REML estimation. Here, time (h) to initiate spore production, the number of spores produced ml^{-1}, and the number of repeated zoospore emergence cycles were used as response variables, with phylogenetic clade included as a fixed factor and strain as a random effect nested within clade, to account for the among-strain variability within clades. Residual distributions were specified as $N(0, \sigma^2)$ for the number of repeated zoospore emergence cycles and the time (h) to initiate spore production, and Tweedie(μ, ϕ, p) for the number of spores produced ml^{-1}. The Tweedie distribution was chosen here, as Bürker chamber counts were converted to continuous spore concentration estimates displaying right-skewed, heteroscedastic behaviour.

Because the strains clustered into 3 phylogenetic clades, with 7 strains belonging to clade I, and clades II and III being singletons, we first evaluated the relative variation within clade I versus between clades by comparing the variance of strain-level random effects within clade I to the fixed effect clade differences. To further assess the proportion of phenotypic variation explained by the differences among phylogenetic clades versus individual strains, we adopted a variance-partitioning approach utilising marginal (R^2_m) and conditional R^2 (R^2_c) values (Nakagawa & Schielzeth 2013, Nakagawa et al. 2017). As R^2 values cannot be directly defined for GLMMs fitted with non-independent residual correlation structures (Nakagawa & Schielzeth 2013, Nakagawa et al. 2017), the R^2 values for hyphal growth were estimated from timepoint-specific GLMMs fitted with Student's *t* residual distribution. Here, log-transformed hyphal area (mm^2) was used as response variable, with phylogenetic clade included as a fixed factor and strain as a random effect nested within clade. For all GLMMs, significance of the fixed effects was tested using a Type III Wald χ^2 test.

Lastly, phenotypic associations between traits were assessed using Type II regression via standardised major axis analysis (SMA), because all variables contained error. Strain-level means were calculated for hyphal growth (mm^2) at 48 h, time (h) to initiate sporulation, number of spores produced ml^{-1}, and number of repeated zoospore emergence cycles. SMA regressions were then used to test pairwise associations among sporulation timing and growth; number of spores produced and growth; number of spores produced and sporulation timing; repeated zoospore emergence and growth; and repeated zoospore emergence and sporulation timing. The area of hyphal growth at 48 h was used here because the hyphae were incubated for 48 h during the sporulation experiment.

All statistical analyses were performed in RStudio, using R v4.5.2 (R Core Team 2025) with $\alpha = 0.05$. GLMMs and Wald χ^2 tests were implemented using the package glmmTMB (Brooks et al. 2017, McGillycuddy et al. 2025), and SMA analysis using the package smatr (Warton et al. 2012). GLMMs were visually inspected for their residual distributions, uniformity, outliers, and dispersion using the DHARMa package (Hartig 2024). R^2_m and R^2_c values (Nakagawa & Schielzeth 2013, Nakagawa et al. 2017) were obtained using the package performance (Lüdecke et al. 2021). Model results were plotted with ggplot2 (Wickham 2016) and patchwork (Pedersen 2025).

3. RESULTS

3.1. Phylogenetic analysis

The strains clustered into 3 distinct clades (I–III) (≥99% bootstrap support) on the phylogenetic tree, all of which were clearly separated from the reference strain CBS 223.65 clade IV (Fig. 1). Seven out of the 9 strains (VH171, VH62, VH28, VH112, VH102, VH162, VH10) clustered into clade I, whereas strains VH70 and VH194 formed their own singleton clades II and III, respectively. Because clades II and III contain only 1 strain each, clade-level patterns should be interpreted cautiously, as they reflect differences of individual strains in relation to clade I.

3.2. Effect of time under laboratory maintenance on phenotypic traits

Maintenance time in laboratory conditions did not systematically influence all the measured variables; however, time (h) to initiate spore production and number of spores produced ml^{-1} showed a statistically significant association with the number of days maintained under laboratory conditions (Table 2, Fig. 2). Contrary to expectation, the predicted time to start sporulation decreased by 0.00555 (±0.00241 SE) h d^{-1} under laboratory maintenance (Type II Wald $\chi^2 = 5.32$, df = 1, p = 0.021), and predicted spore numbers increased by 17.572 (±8.578) spores ml^{-1} d^{-1} under laboratory maintenance (Type II Wald $\chi^2 = 4.20$, df = 1, p = 0.0405) (Fig. 2).

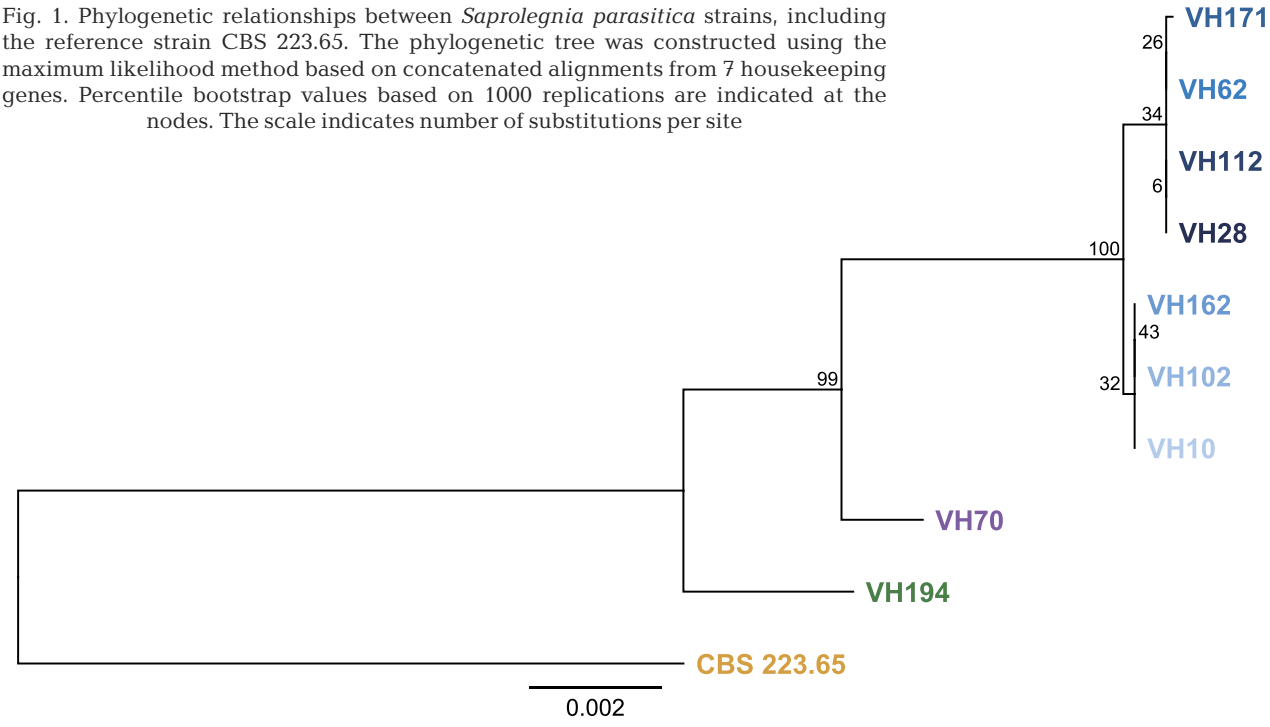

Fig. 1. Phylogenetic relationships between *Saprolegnia parasitica* strains, including the reference strain CBS 223.65. The phylogenetic tree was constructed using the maximum likelihood method based on concatenated alignments from 7 housekeeping genes. Percentile bootstrap values based on 1000 replications are indicated at the nodes. The scale indicates number of substitutions per site

Table 2. Estimated linear effects of days under laboratory maintenance on strain-level means of hyphal growth at 48 h, time to initiate sporulation, number of spores produced, and number of repeated zoospore emergence cycles. Estimates and standard errors (SE) are on the original response scales

	Estimate	SE	*z*-value	p-value
Hyphal area (mm^2) at 48 h	0.187	0.163	1.150	0.25
Time (h) to initiate spore production	−0.00555	0.00241	−2.306	0.0211
Number of spores produced	17.572	8.578	2.048	0.0405
Number of repeated zoospore emergence cycles	−0.000180	0.000760	−0.237	0.813

3.3. Hyphal growth differences

Phylogenetic clade (Type III Wald χ^2 = 15.0, df = 2, p = 0.00054), timepoint (Type III Wald χ^2 = 922.0, df = 2, p < 0.0001), and their interaction (Type III Wald χ^2 = 28.52, df = 4, p < 0.0001) all significantly affected hyphal growth (Fig. 3), indicating time-point-dependent clade differences (for strain-level random intercepts, see Table A3). At 24 h, strains VH70 and VH194 showed significantly reduced growth with estimated hyphal areas being 232 (±58) and 202 (±53) mm^2, respectively, relative to strains within clade I at 355 (±29) mm^2. Strain-level variation within clade I was negligible (Table 3), i.e. strains within clade I had practically identical hyphal areas. Subsequently, the phylogenetic clustering explained 21% (R^2_m = 0.213) of the variation in the hyphal area (mm^2), with no additional variation explained by strain-level differences. At 48 h, strains VH70 and VH194 still showed reduced growth with estimated hyphal areas being 1351 (±214) and 1261 (±199) mm^2, respectively, relative to strains within clade I at 1845 (±100) mm^2, although VH70 was marginally non-significant (Table 3, Fig. 3). Variation between clade I and strains VH70 and VH194 was 2 to 3 times larger than within clade I (Table 3). Phylogenetic clustering explained 14% (R^2_m = 0.138) of the variation in the hyphal area (mm^2), with differences between all strains explaining an additional 13% (R^2_c = 0.264). At 72 h, VH70 and VH194 still displayed reduced growth (Fig. 3), with estimated hyphal areas being 4060 (±564) and 3790 (±531) mm^2, respectively, compared to the estimated hyphal area of clade I at 4700 (±227) mm^2, but differences became statistically non-significant as variation within clade I surpassed the variation between clade I and strains VH70 and VH194 (Table 3).

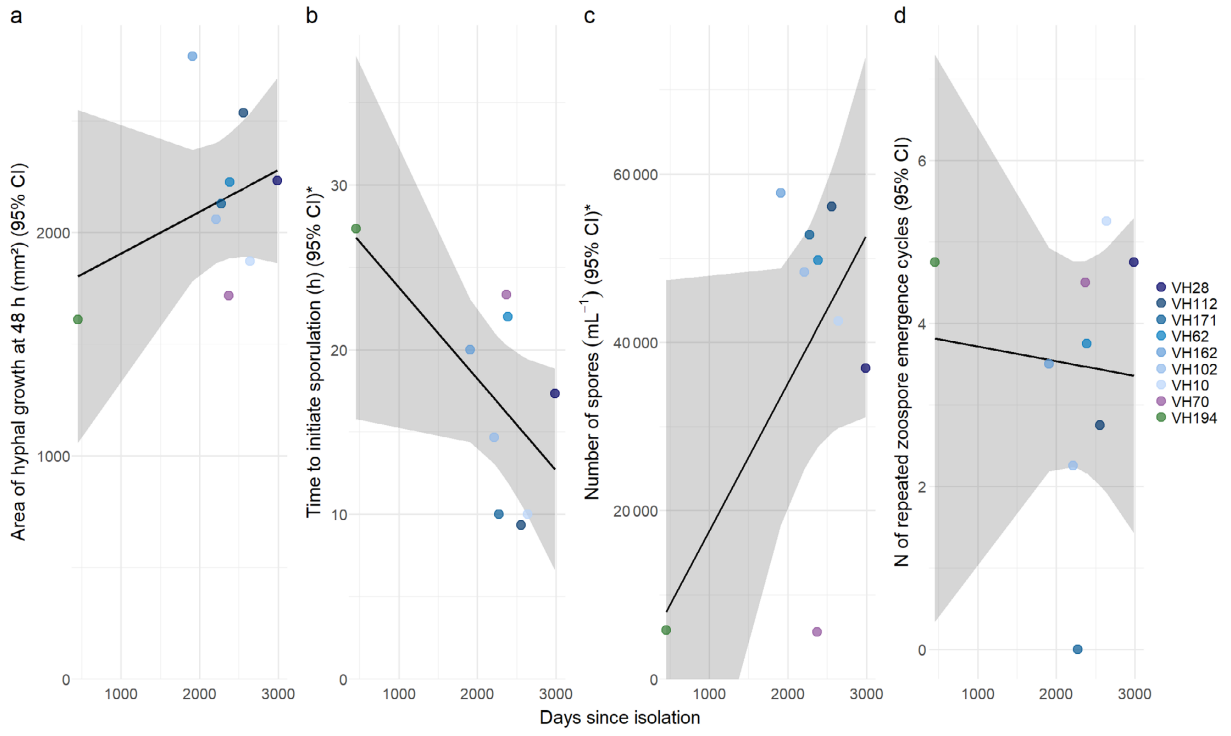

Fig. 2. Predicted effect of time in laboratory maintenance (days since isolation) on (a) *Saprolegnia parasitica* hyphal growth, (b) time to initiate sporulation, (c) number of spores produced, and (d) number of repeated zoospore emergence cycles. Points indicate strain-level means; *statistically significant ($\alpha = 0.05$)

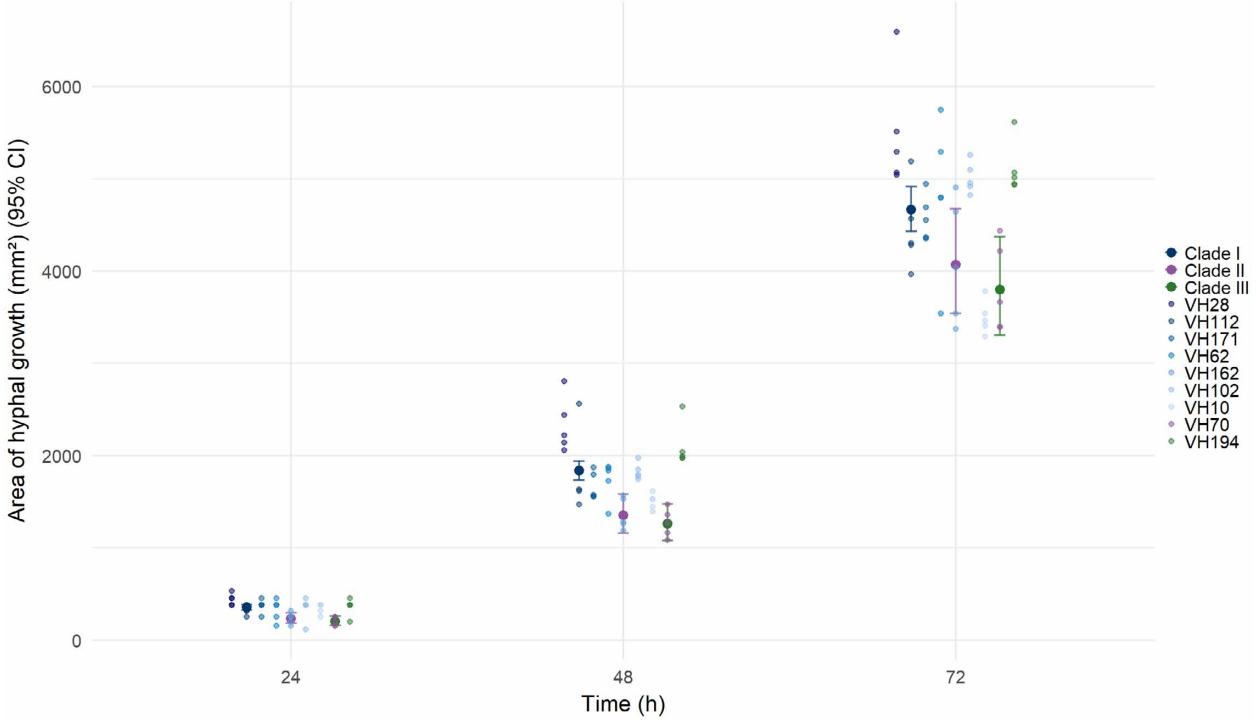

Fig. 3. Predicted growth among *Saprolegnia parasitica* phylogenetic clades (large points with confidence intervals). Small points indicate actual measurements of individual isolates. Clades II and III are singletons formed by strains VH70 and VH194, respectively

Table 3. Strain-level variance (σ^2) within clade I, the repeatability of the strain-level replicates (intra-class correlation) and estimated effects of singleton clades II and III on hyphal growth at 24, 48, and 72 h, time to initiate sporulation, number of spores produced, and number of repeated zoospore emergence cycles, from GLMMs with isolate as a random effect. Estimates, standard errors (SE), and isolate-level σ^2 within clade I are on the log-scale for hyphal area at 24, 48, and 72, and the number of spores ml^{-1}. For the time to initiate sporulation and the number of repeated zoospore emergence cycles estimates, SE and σ^2 are on the original scales (hours and counts, respectively); na: not applicable

Response variable	Strain-level σ^2 within clade I	Intra-class correlation	Effect	Estimate	SE	*z*-value	p-value
Hyphal area (mm^2) 24 h	3.004×10^{-21}	na	Clade II	−0.350	0.0810	−4.32	<0.0001
			Clade III	−0.494	0.112	−4.4	<0.0001
Hyphal area (mm^2) 48 h	0.0127	0.680	Clade II	−0.269	0.138	−1.95	0.0511
			Clade III	−0.320	0.136	−2.35	0.0189
Hyphal area (mm^2) 72 h	0.0156	0.730	Clade II	−0.132	0.152	−0.87	0.384
			Clade III	−0.203	0.148	−1.37	0.171
Time (h) to initiate sporulation	21.900	0.760	Clade II	8.571	5.542	1.547	0.122
			Clade III	12.571	5.542	2.268	0.0233
Number of spores ml^{-1}	0.0156	0.020	Clade II	−2.176	0.256	−8.48	<0.0001
			Clade III	−2.127	0.253	−8.40	<0.0001
Number of repeated zoospore emergence cycles	2.901	0.904	Clade II	1.321	1.869	0.707	0.480
			Clade III	1.5714	1.8690	0.841	0.400

3.4. Sporulation differences

The studied strains varied in their sporulation timing, spore production, and repeated zoospore emergence (Fig. 4). Strain VH194 took an estimated 27.3 (±5.9) h to initiate sporulation, which was significantly slower in relation to strains within clade I that took an estimated 14.8 (±2.0) h. With an estimated time of 23.3 (±5.9) h, the behaviour of VH70 was not significantly different from that of the strains within clade I (Table 3, Fig. 4a). Strain-level variation within clade I was substantial (Table 3), and correspondingly, the phylogenetic clade explained 40% of the variation ($R^2_m = 0.395$), while differences among individual strains explained

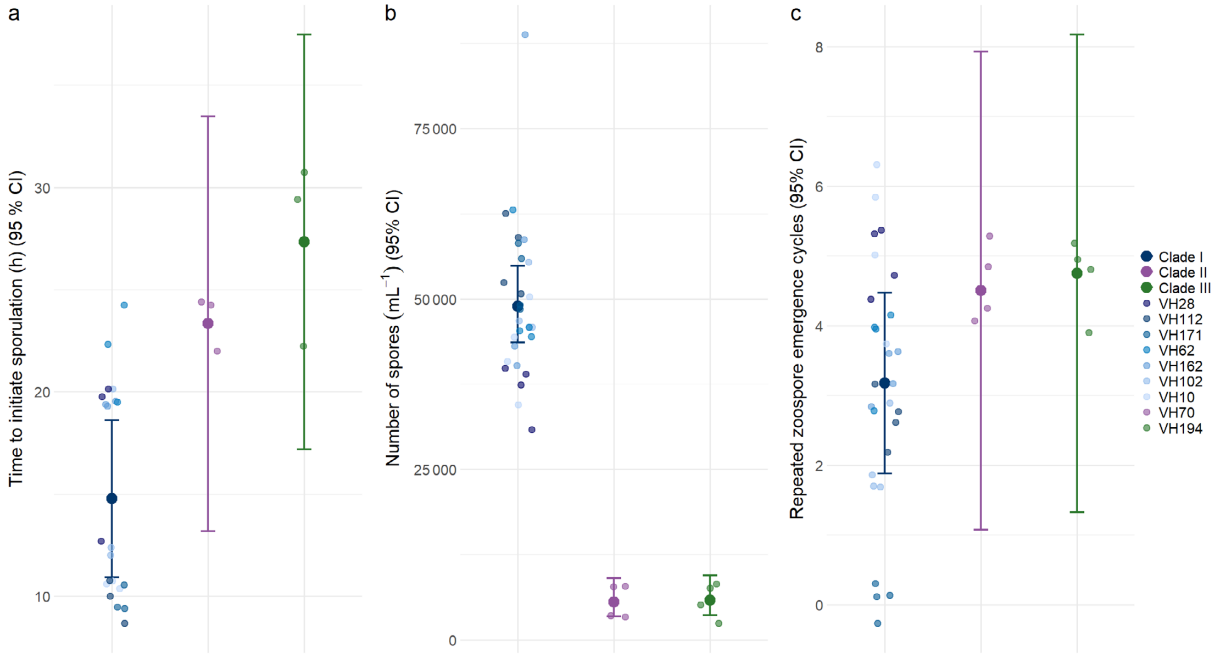

Fig. 4. (a) Predicted time to initiate sporulation, (b) number of spores produced, and (c) number of repeated zoospore emergence cycles among *Saprolegnia parasitica* phylogenetic clades (large points with confidence intervals). Small points indicate actual measurements of individual isolates. Clades II and III are singletons, formed by strains VH70 and VH194, respectively

an additional 45% (R^2_c = 0.852). Strains VH70 and VH194 produced significantly less spores than strains within clade I (Table 3, Fig. 4b), with strains within clade I producing 48 900 (±2900) spores ml^{-1} compared to 5540 (±1460) spores ml^{-1} for VH70 and 5840 (±1520) spores ml^{-1} for VH194. Strain-level variation within clade I was substantially smaller than variation between clade I and strains VH70 and VH194 (Table 3). Subsequently, phylogenetic clustering explained 94% of the variation (R^2_m = 0.940), while differences among all strains only explained an additional 2% (R^2_c = 0.957). Strains VH70 and VH194 did not differ from clade I in the number of repeated zoospore emergence cycles (Table 3, Fig. 4c). Substantial strain-level variation within clade I (Table 3) resulted in the phylogenetic clustering explaining only 10% of the variation (R^2_m = 0.102), while differences among individual strains explained an additional 81% (R^2_c = 0.914).

3.5. Associations between growth and sporulation traits

Type II regression analysis revealed mixed patterns of phenotypic associations among the measured traits. Time (h) to initiate sporulation and area (mm^2) of hyphal growth at 48 h showed no statistically significant association (R^2 = 0.113, p = 0.376). In contrast, the number of spores produced ml^{-1} was positively associated with hyphal growth at 48 h (R^2 = 0.665, p = 0.00737) and negatively associated with time (h) to initiate sporulation (R^2 = 0.462, p = 0.0441). Hence, strains that grew faster and started sporulation earlier also produced more spores. Number of repeated zoospore emergence cycles showed no significant associations with hyphal growth (R^2 = 0. 0.102, p = 0.402) or sporulation timing (R^2 = 0.236, p = 0.185), indicating that this trait varies independently of the other measured traits.

4. DISCUSSION

The studied *Saprolegnia parasitica* strains displayed statistically significant and repeatable differences in hyphal growth and sporulation traits *in vitro*. The observed phenotypic variation was partly explained by both phylogenetic clustering (clades) and differences among individual strains, suggesting that the variation reflects a genetic basis (Erdei et al. 2023). Additionally, the observed positive associations between number of spores produced, sporulation timing, and hyphal growth imply potential covariation in pathogenicity traits (Matthews et al. 2021, Pavić et al. 2022, Erdei et al. 2023). Hyphal areas of *S. parasitica* strains in this study were comparable with previously reported radial growth rates equivalent to 2463–2827 mm^2 (Songe et al. 2014) and 3421–3848 mm^2 (Thoen et al. 2011) on agar after 72 h at 20°C. Conversely, 89% of the strains went through multiple repeated zoospore emergence cycles, compared to the previously reported 21% (Bangyeekhun et al. 2003). The growth and sporulation of another closely related and highly problematic species, *S. diclina*, were not investigated here, but *S. parasitica* and *S. diclina* differ in their physiological responses to environmental factors such as temperature and pH, which influence growth dynamics (Kitancharoen et al. 1996, van den Berg et al. 2013).

Extended *in vitro* subculturing can affect microbial phenotypes, including those of oomycetes and *Saprolegnia* spp., although with variable effects that can be difficult to predict (Songe et al. 2014). Here, the observed significant association of number of days under laboratory maintenance with the time to initiate sporulation and the number of spores produced was apparently driven by the most recently extracted and genetically distinct strain VH194. For the number of spores produced, visual inspection of the model predictions showed an opposing trend within clade I, where the predicted spore numbers decreased as the time under laboratory maintenance increased; however, this observation does not affect the interpretation of the results in terms of functional differences between strains VH70 and VH194 and the other 7 strains within clade I.

Previous studies have not been able to show clear associations between MLST characterisation and host species or geographical origin (Ravasi et al. 2018, Engblom et al. 2023). Here, strain VH194, which originated from the geographically separated, most northern aquaculture facility, was also genetically distinct and furthest away from the other strains; on the other hand, more reliable inspection of such links would call for a more comprehensive sample size. However, the phylogenetic clustering was reflected in the virulence-related physiological traits, as differences between stains VH70 and VH194 and clade I explained between 5 and 94% of the variation in growth and sporulation. Nevertheless, differences among all strains accounted substantially for the observed variation, indicating functional variation within common genetic lineages as well as between them. Considering that growth and sporulation are central traits underlying virulence and transmission, respectively (Walker & van West 2007, Bruno et al. 2011), their variation among strains could

indicate microevolutionary adaptations to local conditions (André & Hochberg 2005, Kennedy et al. 2016). However, our laboratory setting and genetic framework did not allow differentiation of random genetic drift from adaptive differences.

The genetically distinct strains VH70 and VH194 had the slowest growth, produced the fewest spores, and took the longest time to initiate sporulation. Correspondingly, faster growth and rapid sporulation was associated with a higher number of spores produced across all strains. These positive phenotypic associations between growth and sporulation traits suggest that the abundance of hosts in aquaculture environments may simultaneously favour high virulence and transmission capacities, rather than establishing trade-offs between the traits (André & Hochberg 2005, Alizon et al. 2009). The single strain that originated from a wild fish clustered within clade I, which could either suggest spread of potential high-virulence strains from aquaculture facilities to natural waterbodies or natural occurrence of common genetic lineages across environments.

Limitations of this study include the usage of only 9 strains that were all extracted from salmonids, leaving broader host diversity (e.g. cyprinids) unexplored and potentially underestimating total variation. Seven out of the 9 strains clustered into the phylogenetic clade I, and 2 strains formed their own singleton clades II and III. Thus, for these 2, clade and strain level effects cannot be disentangled, and their phenotypic differences with respect to other strains cannot be attributed to clade identity alone. We also cannot infer whether the phylogeny formed based on common housekeeping genes (Ravasi et al. 2018, Engblom et al. 2023) reflects any of the true genetic variation in virulence and transmission traits. Lastly, although allowing comparisons with previous studies utilising the same growth and sporulation methodologies, the *in vitro* nature of the assays omits direct host–pathogen interactions such as immune evasion or tissue specificity (Tedesco et al. 2022), and the incubation temperature of 20°C exceeds typical epidemic conditions (often 10–15°C in cold-water salmonids) (Kitancharoen et al. 1996, Janhunen et al. 2019), possibly inflating growth rates.

In summary, observations of significant phenotypic variation and the positive covariation in potential virulence and transmission traits among *S. parasitica* strains suggest that common genetic lineages are not functionally equivalent. Along with earlier reports of pronounced phenotypic and virulence diversity, these findings underscore the importance of monitoring not only the presence of *Saprolegnia* species but also the composition of genetic strains within and among aquaculture facilities. Such variation may influence outbreak dynamics and pathogen evolution, increasing the risk of potential rapid virulence changes and further spread of highly virulent strains within and across aquaculture environments. To further understand these processes, detailed genomic studies are urgently required.

Data availability statement. The data that support the findings of the study is available at https://doi.org/10.5281/zenodo.18953216.

Author contributions. O.L.: Conceptualisation (equal), data curation (lead), formal analysis (lead), funding acquisition (lead), methodology (equal), writing—original draft preparation (lead), writing—review and editing (equal). J.H.: Conceptualisation (equal), investigation (lead), methodology (equal), writing—original draft preparation (supporting), writing—review and editing (equal), supervision (equal). T.K.: Conceptualisation (equal), investigation (supporting), methodology (equal), writing—review and editing (equal), supervision (equal). M.J.: Conceptualisation (equal), writing—review and editing (equal), supervision (equal). A.V.: Conceptualisation (equal), writing—original draft preparation (supporting), writing—review and editing (equal), funding acquisition (equal), supervision (lead).

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Appendix.

Table A1. Composition and preparation of PG-1 agar and supplements used for *Saprolegnia parasitica* cultivation

Component	Amount	Notes/supplier
PG-1 agar base	2000 ml	Prepared in-house; autoclaved 121°C 120 min, cooled to ~48°C before supplementation
Bacto peptone	6 g	Becton Dickinson
D-(+)-glucose monohydrate	12 g	Sigma-Aldrich
Bacto agar	24 g	VWR
Milli-Q water	1600 ml	—
Salt solution	200 ml	$MgCl_2 \cdot 6H_2O$ 3.4 g, $CaCl_2 \cdot 2H_2O$ 2.9 g, $FeCl_3 \cdot 6H_2O$ 0.4 g, KCl 7.4 g, Titriplex III 1.1 g; autoclaved 121°C 15 min, stored at 4°C
Phosphate buffer	200 ml	Na_2HPO_4 0.0067 M + $NaH_2PO_4 \cdot 2H_2O$ 0.067 M; mixed, autoclaved 121°C 15 min, stored at 4°C
PG-1 agar (final)	2000 ml	Cooled base combined with salt solution and phosphate buffer

Table A2. GenBank accession numbers for the 7 loci for each isolate, used for the phylogenetic analysis

Gene	Isolate	GenBank	Gene	Isolate	GenBank	Gene	Isolate	GenBank
ALTS1	VH10	PZ161730	NAD1	VH10	PZ161727	TUBB	VH10	PZ161724
	VH102	PZ161702		VH102	PZ161699		VH102	PZ161696
	VH112	PZ161695		VH112	PZ161692		VH112	PZ161689
	VH162	PZ161688		VH162	PZ161685		VH162	PZ161682
	VH171	PZ161681		VH171	PZ161678		VH171	PZ161675
	VH194	PZ161674		VH194	PZ161671		VH194	PZ161668
	VH28	PZ161723		VH28	PZ161720		VH28	PZ161717
	VH62	PZ161716		VH62	PZ161713		VH62	PZ161710
	VH70	PZ161709		VH70	PZ161706		VH70	PZ161703
COX1	VH10	PZ161729	RPB2	VH10	PZ161726			
	VH102	PZ161701		VH102	PZ161698			
	VH112	PZ161694		VH112	PZ161691			
	VH162	PZ161687		VH162	PZ161684			
	VH171	PZ161680		VH171	PZ161677			
	VH194	PZ161673		VH194	PZ161670			
	VH28	PZ161722		VH28	PZ161719			
	VH62	PZ161715		VH62	PZ161712			
	VH70	PZ161708		VH70	PZ161705			
GLUT	VH10	PZ161728	SHMT	VH10	PZ161725			
	VH102	PZ161700		VH102	PZ161697			
	VH112	PZ161693		VH112	PZ161690			
	VH162	PZ161686		VH162	PZ161683			
	VH171	PZ161679		VH171	PZ161676			
	VH194	PZ161672		VH194	PZ161669			
	VH28	PZ161721		VH28	PZ161718			
	VH62	PZ161714		VH62	PZ161711			
	VH70	PZ161707		VH70	PZ161704			

Table A3. Strain-level random intercepts for all models

Model	Clade	Strain	Random intercept
Hyphal area (mm^2) 24 h	Clade I	VH28	2.321036×10^{0}
		VH112	-4.899965×10^{0}
		VH171	-4.298215×10^{0}
		VH62	6.533286×10^{0}
		VH162	4.728036×10^{0}
		VH102	-8.596429×10^{-2}
		VH10	-4.298215×10^{0}
	Clade II	VH70	1.948568×10^{-30}
	Clade III	VH194	4.676343×10^{-30}
Hyphal area (mm^2) 48 h	Clade I	VH28	7.467744×10^{-3}
		VH112	1.018334×10^{-1}
		VH171	-1.020528×10^{-2}
		VH62	-6.010417×10^{-2}
		VH162	1.835189×10^{-1}
		VH102	-7.004559×10^{-2}
		VH10	-1.524649×10^{-1}
	Clade II	VH70	1.137359×10^{-28}
	Clade III	VH194	$-1.686747 \times 10^{-22}$
Hyphal area (mm^2) 72 h	Clade I	VH28	6.361288×10^{-2}
		VH112	7.764280×10^{-2}
		VH171	5.412508×10^{-2}
		VH62	-4.811442×10^{-2}
		VH162	1.208535×10^{-1}
		VH102	-1.658406×10^{-2}
		VH10	-2.515358×10^{-1}
	Clade II	VH70	$-1.368225 \times 10^{-27}$
	Clade III	VH194	5.175258×10^{-26}
Number of repeated zoospore emergence cycles	Clade I	VH28	1.530966×10^{0}
		VH112	-4.175361×10^{-1}
		VH171	-3.096726×10^{0}
		VH62	5.567148×10^{-1}
		VH162	3.131520×10^{-1}
		VH102	-9.046615×10^{-1}
		VH10	2.018091×10^{0}
	Clade II	VH70	$-6.845317 \times 10^{-60}$
	Clade III	VH194	$-2.277110 \times 10^{-45}$
Number of spores ml^{-1}	Clade I	VH28	-1.584025×10^{-1}
		VH112	8.614702×10^{-2}
		VH171	4.692564×10^{-2}
		VH62	9.849721×10^{-3}
		VH162	1.053017×10^{-1}
		VH102	-7.375868×10^{-3}
		VH10	-8.244568×10^{-2}
	Clade II	VH70	$-2.708401 \times 10^{-33}$
	Clade III	VH194	7.334650×10^{-28}
Time (h) to initiate sporulation	Clade I	VH28	2.321036×10^{0}
		VH112	-4.899965×10^{0}
		VH171	-4.298215×10^{0}
		VH62	6.533286×10^{0}
		VH162	4.728036×10^{0}
		VH102	-8.596429×10^{-2}
		VH10	-4.298215×10^{0}
	Clade II	VH70	1.948568×10^{-30}
	Clade III	VH194	4.676343×10^{-30}

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