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Spatial variation in species composition of Saprolegnia, a parasitic oomycete of amphibian eggs, in Scotland

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Abstract :

Parasitic water moulds in the genus Saprolegnia cause mortality of amphibian embryos and reduced size at metamorphosis, leading to increased adult mortality. Most studies of virulence have focused on only a single Saprolegnia species, but the Saprolegnia species associated with amphibian eggs and their distributions are not well known. This study aimed to investigate the distribution of amphibian-associated water moulds in Scotland. In particular, we asked the questions: i) Does Saprolegnia species composition vary between sites?; and ii) Is presence of Saprolegnia related to environmental parameters? Common frog (Rana temporaria) eggs with evidence of Saprolegnia infection were sampled from ten sites, cultured, and the 28S region of the rDNA array sequenced. Thirteen samples isolated from four sites were identified as members of the Saprolegniaceae and the ITS region of these samples were subsequently sequenced to further resolve species identification. Four species of Saprolegnia were found in total, with one or two species of Saprolegnia present in each of four sites. S. diclina was the most common species identified and was found at three of the four sites. Acidity was significantly lower and altitude significantly higher at sites where Saprolegniaceae were present. Therefore, R. temporaria eggs in different pools are subject to infection by different, and in some instances more than one, species of Saprolegnia. Overall, our findings suggest that future studies of virulence need to consider the effect of multiple Saprolegnia species within a site as well as the population of origin of the amphibian host.

Keywords : disease ecology, Rana temporaria, Scotland, water mould

INTRODUCTION

Biodiversity is declining worldwide at an unprecedented rate (Barnosky *et al.* 2011), with amphibians showing higher extinction rates than any other vertebrate taxa (Stuart *et al.* 2004). Causes of the observed amphibian declines include direct anthropogenic effects such as habitat conversion and loss, overexploitation and introduction of invasive species (Blaustein & Kiesecker 2002). However, up until the late 1990s there were also many documented cases of "enigmatic" declines, where suitable habitat remained and the causes of declines were not fully understood (Stuart *et al.* 2004). Such declines have increasingly been linked to *Batrachochytrium dendrobatidis* (Bd), a fungus that causes chytridiomycosis in amphibians (Fisher *et al.* 2009), and Bd has become the focus of most epidemiological studies in amphibians since its discovery (Duffus 2009). However, increased mortality in amphibians has also been linked to other diseases including ranavirus (Cunningham *et al.* 1996), redleg disease (Bradford 1991), and water moulds in the genus *Saprolegnia* (Bragg 1962; Blaustein *et al.* 1994).

Water moulds (genus *Saprolegnia*) are ubiquitous freshwater and soil oomycetes that show both saprobic and parasitic feeding strategies (Romansic *et al.* 2006). *Saprolegnia* species infect a range of hosts and cause saprolegniasis in fish, a disease of significant economic importance to the aquaculture industry (Vanwest 2006). In amphibians, *Saprolegnia* infects eggs and larvae, passing from one individual to another via free-swimming zoospores or, more commonly, via direct contact with growing hyphae (Robinson *et al.* 2003). Non-viable eggs are more readily colonised by *Saprolegnia* than viable eggs, but infections can rapidly spread to adjacent live eggs, causing embryo mortality (Robinson *et al.* 2003), or a reduced size at metamorphosis (Uller *et al.* 2009) and thus increased adult mortality (Altwegg & Reyer 2003).

Although extensive research has investigated the effects of parasitism by *Saprolegnia* on amphibian embryos (Romansic *et al.* 2009; Ruthig 2009; Uller *et al.* 2009), less attention has been paid to the species of *Saprolegnia* that infect amphibian embryos in the wild and whether species composition varies by site (but see Petrisko *et al.* 2008). Furthermore, virulence studies have focussed predominantly on the effect of a single *Saprolegnia* species (Sagvik *et al.* 2008; Romansic *et al.* 2009;

Uller *et al.* 2009), without knowledge of whether multiple *Saprolegnia* species cause amphibian infection in the wild. Molecular methods for species-level identification offer the opportunity to examine which species of *Saprolegnia* are found on amphibian eggs (Petrisko *et al.* 2008), a question that has previously been neglected due to the challenging nature of morphological species identification in this group (Ault *et al.* 2012).

The common frog (*Rana temporaria*) is found throughout Europe and is an explosive breeder, with multiple egg masses joining to form communal egg mats, which are thought to regulate and maintain temperature conditions for growing embryos (Håkansson & Loman 2004). However, communal spawning puts *R. temporaria* eggs at increased risk of hyphal spread of *Saprolegnia* between individuals (Kiesecker & Blaustein 1997). Indeed *Saprolegnia* has been found to cause mortality in *R. temporaria* eggs by spreading from infected dead eggs to live eggs (Robinson *et al.* 2003). In Scotland, *R. temporaria* breed in a wide variety of water bodies, thus experiencing a range of different environmental conditions (Inns 2009), making them ideal for studying the relationship between *Saprolegnia* species presence and environmental conditions. Furthermore, west central Scotland has relatively low levels of intensive agriculture (Swan *et al.* 1994), avoiding confounding interactions between water mould presence, amphibian susceptibility and pollutants that have been found elsewhere (Romansic *et al.* 2006).

The overall aim of this study was to investigate the distribution of water moulds sampled from *R. temporaria* egg masses in Scotland. In particular, we asked the questions: 1) Does *Saprolegnia* species composition vary between sites?; and 2) Is presence of *Saprolegnia* related to environmental parameters?

METHODS

Sampling

R. temporaria eggs that putatively showed evidence of infection by water mould (identified as a white “cotton wool” covering the surface of the egg; Fernández-Benéitez *et al.* 2008) were collected from ten sites across central Scotland during the 2012 breeding season (March-April; for locations and site name abbreviations see Figure 1). Sites represented a range of habitats, including: urban, semi-rural and rural; temporary and permanent water bodies; and water bodies of different sizes from pool,

pond and marsh to loch (Table 1). Three potentially infected eggs were collected from each of five egg masses per site. Eggs were stored in individual microcentrifuge tubes and transported to the laboratory in cool bags. At each site the water parameters pH (to 0.01 pH), conductivity (to 1 µS cm^{-1}) and total dissolved solids (to 1 ppm) were recorded using an HI 98129 Waterproof pH/EC/TDS/Temperature Tester (Hanna instruments, Leighton Buzzard).

In the laboratory, a section of white water mould (roughly $1mm^3$) was removed from each egg, ensuring that no egg tissue or jelly capsule remained attached, and rinsed with distilled water containing 100mg l^{-1} of penicillin C to reduce bacterial contamination (Fernández-Benéitez *et al.* 2011). The water mould was then placed on a glucose-peptone-salts (GYPS) agar plate containing 5g l^{-1} glucose, 0.5g l^{-1} peptone, 0.5g l^{-1} KH2PO4, 0.05g l^{-1} yeast extract and 0.15g l^{-1} MgSO4.7H2O (Beakes & Ford 1983). Antibiotics (10ml l^{-1} of ampicillin and 5ml l^{-1} of chloramphenicol) were also added to agar plates to prevent bacterial growth (Fernández-Benéitez *et al.* 2011). Plates were sealed and maintained at room temperature (around 23°C) until hyphae growth covered half of the plate surface, at which point hyphae from the edge of the mycelium mat were transferred to a second plate for growth to continue. After ten days of growth, peripheral hyphae were transferred to a 1.5ml microcentrifuge tube containing 500µl of liquid GYPS media and maintained in a thermal cabinet at room temperature (23°C) for 72 hours (Cenis 1992).

DNA extraction and sequencing

DNA extraction was carried out following the protocol in Cenis (1992): microcentrifuge tubes containing mycelium mats and liquid media were centrifuged for 10 minutes at 13,000 rpm, excess liquid media was removed and 500µl of TE buffer added before centrifuging again for five minutes; from this point extractions followed a standard DIGSOL extraction method (Nicholls *et al.* 2000). DNA was resuspended in 30µl of TE buffer.

To assess the diversity of eukaryotic microbial species cultured in the samples, a 622 bp section of the 28S rRNA region, including the hypervariable stem and loop regions between helices C1 and D2, was amplified using eukaryote specific primers: C1 (5°-ACCCGCTGATTTAAGCAT-3°) and D2 (5° - TCCGTGTTTCAAGACGG-3°) (Leclerc 2000; Hulvey *et al.* 2007). Polymerase chain reactions (PCR) were performed in 20µl reaction volumes containing: 2.5 mM $MgCl_2$ (Invitrogen), 1 x

PCR Buffer (Invitrogen), 0.2 mM dNTP (Invitrogen), 0.1 μM forward primer, 0.1 μM reverse primer, 0.5 units of Taq polymerase (Invitrogen) and 1 μl of DNA template. Initial denaturisation took place at 94°C for 3 minutes; followed by 35 cycles of 94°C for 30 seconds, 54°C for 30 seconds and 72°C for 90 seconds; with a final extension step of 72°C for 10 minutes. Amplified samples were cleaned with ExoSAP-IT (USB, Cleveland), according to the manufacturer's instructions, and sent to the GenePool core genomics facility at the University of Edinburgh, where they were sequenced on an ABI 3730 automated sequencer. To resolve ambiguities encountered surrounding *Saprolegni*a species identification using the 28S markers (please see results), samples identified as *Saprolegnia* species using the 28S region were further investigated by amplifying a 514bp section of the ribosomal internal transcribed spacer (ITS) region using the fungal primers ITS1 (forward 5'-3' TCCGTAGGTGAACCTGCGG) and ITS4 (reverse 5'-3' TCCTCCGCTTATTGATATGC) (Hulvey *et al.* 2007). PCR and sequencing proceeded as before.

Species composition between sites

Sequences were aligned and base-calling errors corrected using Sequencher v4.5 (Gene Codes Corporation, Ann Arbour), and then matched to published sequences in the NCBI Genbank database using megaBLAST. Species names were assigned to samples based on the maximum percentage of identical nucleotides between the sample and reference sequences within the alignment length (Max ident), when the percentage of the sample sequence covered by the reference sequence (Query coverage) was at least 90%.

All reference sequences in the genus *Saprolegnia* matched using megaBLAST for the 28S sequences were downloaded from Genbank (Accession numbers HQ665061, HQ665062, HQ665127, HQ665142, HQ665197, HQ665214, HQ665253, HQ665270: Robideau *et al.* 2011; AF119613, AF119616: Riethmüller *et al.* 2000; AF218166: Leclerc 2000). Reference sequences were aligned to the sample 28S sequences identified as members of the *Saprolegniaceae* using Sequencher v4.5. To visualise differences among the sample and reference sequences, a maximum-likelihood phylogenetic tree was constructed using the Tamura-Nei model of evolution, with 500 bootstrap replications, as implemented in MEGA v5.2.1 (Tamura *et al.* 2011). The closely related *Achlya conspicua* (a

Saprolegniaceae) and *Albugo candida* (an oomycete causing white rust in plants) were used as outgroups (Accession numbers HQ643092 and HQ643110, respectively: Robideau *et al.* 2011).

Presence in relation to environmental parameters

To evaluate whether isolation of *Saprolegnia* from a site (response variable, considered as a categorical variable of present or absent) significantly varied in relation to the environmental parameters measured, a generalised linear mixed model approach (GLMM) was used with a binomial distribution, as implemented in R v2.12.1 (R core development team). Each model parameter (environmental measure: each considered as a random continuous variable) and interaction between parameters was sequentially removed from the model and an analysis of variance (ANOVA) used to evaluate significance of terms for retention in the final model.

RESULTS

Species composition between sites

In total, 28S sequences were obtained from 72 samples from across the ten sites that showed colony growth and had a match identified using BLAST (Table A1, Appendix). Overall, 24 eukaryote microbial families, consisting of 37 species were identified, including 33 non-*Saprolegnia* species (Table 1). Thirteen samples from four sites were identified as belonging to the *Saprolegnia* family using the 28S region: three samples from BM, four samples from CV, four samples from DM and two samples from QP (Genbank Accession numbers: KF255401-KF255413; Table 2; Figure 1). All samples had at least a 98% match and 90% coverage with Genbank reference sequences (Table A2). However, sequences from multiple species in Genbank showed equally high matches with each sample sequence, resulting in between two and seven species names being assigned to each sample (Table 2). Therefore, a bootstrap consensus tree with maximum-likelihood branch lengths of the 28S sequences was constructed to elucidate the relationship between *Saprolegnia* samples. The tree showed that sequences from samples DM1-4 were identical to each other and to reference sequences identified as *S. mixta* and *S. ferax* (Cluster A; Figure 2). Samples CV1-4, BM2 and 3, and QP2 clustered together but did not cluster with any of the reference sequences (Cluster B; Figure 2). Sample BM1, although most similar to *S. litoralis*, did not cluster with any reference or sample

sequences (the only member of Cluster C; Figure 2). Finally, QP1 was identical to samples identified as *S. unispora*, *S. torulosa* and *S. monilifera*, but not with any other sequences from this study (Cluster D; Figure 2). Bootstrap resolution was not sufficient to draw conclusions about the relationships among clusters, but clusters A and B appeared to be more closely related to one another than to C and D.

Three of the samples that were identified as *Saprolegnia* using the 28S region, failed to be sequenced successfully at the ITS region and ten samples were thus matched to existing species records using Genbank (Table 2). Six of the samples were identified as *S. diclina* (four from CV and two from BM; Sandoval-Sierra *et al.* 2014) and four as *S. ferax* (all from DM; (Sandoval-Sierra *et al.* 2014), with at least a 97% match and 97% coverage to Genbank reference sequences (Tables 2 and A2). Furthermore, although QP2 was not able to be sequenced at the ITS region, due to the 28S sequence of QP2 being identical to BM1, BM2 and CV1-4 (Figure 2) it is possible to infer that this sample is also *S. diclina* (Table 2).

Together, the results from the 28S and ITS sequences showed that four species were present across the four sites: *S. diclina*, *S. ferax, either S. parasitica* or *S. litoralis,* and either *S. monilifera* or *S. unispora* or *S. torulosa* or *S. terrestris* (Table 2). *S. diclina* was the most widespread species, observed at three out of four sites (BM, CV and QP). The other three species were found at only one site each (Table 2). Furthermore, two species of *Saprolegnia* were observed at each of two sites: *S diclina* and *S. parasitica/litoralis* were present at BM, and *S diclina* (cluster B, Figure 2) and *S. monilifera/unispora/torulosa/terrestris* were isolated from QP (Table 2).

Presence in relation to environmental parameters

Only altitude and pH (but not their interaction) significantly changed the deviance of the model when removed (using a chi-squared significance test within the ANOVA; $p<0.01$) and were thus retained, to give a final model of Presence ~ pH + Altitude. Thus, the GLMM of the final model showed that pH and altitude were significant at predicting sites where *Saprolegnia* was present ($t=2.56$ and 2.42, $p=0.04$ and 0.05, respectively). pH and altitude were higher at sites where *Saprolegnia* was present compared to absent (Figure 3).

DISCUSSION

Species composition between sites

Four species of *Saprolegnia* were present within the study system in total, with one or two species of Saprolegnia present in each of four sites (Table 1). *S. diclina* was the most common species identified and was found at three of the four sites sampled. In contrast, a sample identified as either *S. parasitica* or *S. litoralis* was found only at BM, *S. ferax* was only isolated from DM, and a sample identified as either *S. monilifera. S. unispora*, *S. torulosa* or *S. terrestris* was found at QP. At two sites (QP and BM), two species of *Saprolegnia* were found upon *R. temporaria* eggs within the same water body. At QP, *S. diclina* and *S. monilifera/unispora/torulosa/terrestris* were found and at BM, *S. diclina* and *S. parasitica/litoralis* were found. Therefore, *R. temporaria* eggs in different pools are subject to infection by different, and in some instances more than one, species of *Saprolegnia.*

S. diclina was the most common species observed, rather than *S. ferax*, which was previously thought to be the most common *Saprolegnia* species to infect amphibian eggs and thus used in studies of virulence (Romansic *et al.* 2009). Our study adds to a growing body of evidence that *S. diclina* is the most common *Saprolegnia* species to infect amphibian eggs (Petrisko *et al.* 2008; Fernández-Benéitez *et al.* 2008, 2011; Ault *et al.* 2012). However, all the samples from DM were *S. ferax*, showing that *S. ferax* does infect amphibian eggs in Scotland. This finding is in contrast to the findings of Petrisko *et al.* (2008), who did not identify *S. ferax* on infected amphibian eggs in the Pacific Northwest of America, but is in line with the findings of Fernández-Benéitez *et al.* (2011) and Perotti *et al.* (2013) who also isolated *S. ferax* from amphibian eggs. In our study, more than one species of *Saprolegnia* was isolated in each of two sites, showing that multiple species of the water mould co-exist within single sites in Scotland. This is consistent with the work of Ault *et al.* (2012), who isolated multiple species of *Saprolegnia* from a lake in the USA, and Fernández-Benéitez *et al.* (2011), who identified *S. ferax* and *S. diclina* from a single site in Spain However, both these studies only assessed species richness at a single site. Perotti *et al*. (2013) isolated two species from each of two sites in Argentina, although they were unable to identify whether species assemblage varied between these two sites due to non-identification to species level of one isolate. Therefore, our study adds an important finding to the understanding of *Saprolegnia* spatial variation: that *Saprolegnia* species

assemblage can vary between sites, even within a small geographical area (the furthest distance between sites was 90km). Although multiple species of *Saprolegnia* were isolated within a site, our results do not elucidate whether multiple species can infect a single individual, as multiple species of *Saprolegnia* were not identified from a single egg from a site. However, given our findings of multiple species at the same site and the fact that synergistic effects of pathogens can be important in predicting mortality in amphibians (Romansic *et al.* 2011), an important remaining question is whether individuals can be infected by multiple *Saprolegnia* species and what is the effect of infection by multiple species of *Saprolegnia* on amphibian survival.

Presence in relation to environmental parameters

Sites where *Saprolegnia* species were present showed a significantly higher pH (lower acidity) than those where *Saprolegnia* was not isolated (Figure 3). Higher acidity has previously been linked to higher occurrence of *Saprolegnia* infections in a range of amphibian species, including *R. temporaria* and *R. arvalis* (the moor frog), from lakes in the Netherlands (Hartog *et al.* 1986). However, the acidity of the lakes sampled by Hartog *et al.* (1986) was much greater than in this study, with extremely low acidity classed as below pH 4, moderately acid as pH 4-5, and neutral as above pH 5; all the sites sampled in Scotland would be classed as neutral using these categories (Table A3). Furthermore, *R. temporaria* show relatively higher mortality and lower occurrence in high acid environments and are less acid tolerant than their close relative *R. arvalis* (Hartog *et al.* 1986). Therefore, this study has potentially identified a finer scale of pH-related *Saprolegnia* presence and absence in neutral environments than that identified by Hartog *et al.* (1986). Our results show that in relation to a less acid tolerant amphibian species, *R. temporaria*, a relatively higher pH favours *Saprolegnia* growth. The impact of altitude on presence of *Saprolegnia* was also significant, with sites where *Saprolegnia* was present having a higher altitude. To the best of our knowledge, this is the first study that has identified a link between altitude and *Saprolegnia* presence. It is also interesting to note that although the sites where *Saprolegnia* was present varied by habitat type in terms of water body size (marsh, pool, pond or loch) and whether rural, semi-rural or urban, all the sites where *Saprolegnia* was isolated were permanent as opposed to temporary water bodies (Table 1). However, our study relied on culture methods which may be biased towards species that grow best in laboratory conditions (Ault *et al.*

2012). Therefore, research with total embryo-associated DNA, where all DNA is extracted from a collected egg without the use of culture, is needed to further assess whether pH and altitude are important predictors of *Saprolegnia* presence.

Spatial variation in species composition of *Saprolegnia*

Kiesecker *et al.* (2001) found that different isolates of *S. ferax* caused different levels of mortality in western toad embryos, suggesting variability in virulence between strains. Furthermore, Sagvik *et al.*(2008) and Urban *et al.* (2014) identified a genetic component to resistance to infection by *Saprolegnia* in *R. arvalis* and the spotted salamander, *Ambystoma maculatum*, respectively. Therefore, the differences observed between sites in terms of species richness and presence could be due to variability in levels of virulence of different members of the *Saprolegniaceae* (Kiesecker *et al*. 2001) and/or host resistance to water moulds (Sagvik *et al.* 2008; Urban *et al.* 2014). Further research is needed to determine whether host resistance to water moulds varies by breeding site in *R. temporaria*. Overall, our findings suggest that future studies of virulence need to consider the effect of multiple *Saprolegnia* species within a site as well as the population of origin of the amphibian host.

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REFERENCES

Altwegg R, Reyer H-U (2003) Patterns of natural selection on size at metamorphosis in water frogs. *Evolution*, **57**, 872–882.

Ault KK, Johnson JE, Pinkart HC, Wagner RS (2012) Genetic comparison of water molds from embryos of amphibians Rana cascadae, *Bufo boreas* and *Pseudacris regilla*. *Diseases of aquatic organisms*, **99**, 127–137.

Barnosky AD, Matzke N, Tomiya S *et al.* (2011) Has the Earth's sixth mass extinction already arrived? *Nature*, **471**, 51–57.

Beakes GW, Ford H (1983) Esterase isoenzyme variation in the genus *Saprolegnia*, with particular reference to the fish-pathogenic *S. diclina-parasitica* complex. *Journal of general microbiology*, **129**, 2605–2619.

Blaustein AR, Grant Hokit D, O'Hara RK, Holt RA (1994) Pathogenic fungus contributes to amphibian losses in the pacific northwest. *Biological Conservation*, **67**, 251–254.

Blaustein AR, Kiesecker JM (2002) Complexity in conservation: lessons from the global decline of amphibian populations. *Ecology Letters*, **5**, 597–608.

Bradford DDF (1991) Mass Mortality and Extinction in a High-Elevation Population of *Rana muscosa*. *Journal of Herpetology*, **25**, 174–177.

Bragg ANA (1962) Saprolegnia on Tadpoles again in Oklahoma. *The Southwestern Naturalist*, **7**, 79–80.

Cenis JL (1992) Rapid extraction of fungal DNA for PCR amplification. *Nucleic acids research*, **20**, 2380.

Cunningham AA, Langton TE, Bennett PM *et al.* (1996) Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). *Philosophical transactions of the Royal Society B*, **351**, 1539–1557.

Duffus ALJ (2009) Chytrid blinders: what other disease risks to amphibians are we missing? *EcoHealth*, **6**, 335–339.

Fernández-Benéitez MJ, Ortiz-Santaliestra ME, Lizana M, Diéguez-Uribeondo J (2008) *Saprolegnia diclina*: another species responsible for the emergent disease "Saprolegnia infections" in amphibians. *FEMS microbiology letters*, **279**, 23–9.

Fernández-Benéitez MJ, Ortiz-Santaliestra ME, Lizana M, Diéguez-Uribeondo J, Die J (2011) Differences in susceptibility to *Saprolegnia* infections among embryonic stages of two anuran species. *Oecologia*, **165**, 819–826.

Fisher MC, Garner TWJ, Walker SF (2009) Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. *Annual review of microbiology*, **63**, 291–310.

Håkansson P, Loman J (2004) Communal spawning in the common frog *Rana temporaria*–egg temperature and predation consequences. *Ethology*, **110**, 665–680.

Hartog C Den, Christiaans MMC, Heijligers WHC, Leuven RSEW, den Hartog C (1986) Effects of water acidification on the distribution pattern and the reproductive success of amphibians. *Experientia*, **42**, 495–503.

Hillis DM, Bull JJ (1993) An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic biology*, **42**, 182–192.

Hulvey JP, Padgett DE, Bailey JC (2007) Species boundaries within *Saprolegnia* (Saprolegniales, Oomycota) based on morphological and DNA sequence data. *Mycologia*, **99**, 421–429.

Inns H (2009) *Britain's reptiles and amphibians*. WildGuides UK.

Kiesecker JMJ, Blaustein AAR (1997) Influences of Egg Laying Behavior on Pathogenic Infection of Amphibian Eggs. *Conservation Biology*, **11**, 214–220.

Kiesecker JM, Blaustein AR, Miller CL (2001) Transfer of a Pathogen from Fish to Amphibians. *Conservation Biology*, **15**, 1064–1070.

Leclerc M (2000) Taxonomic and phylogenetic analysis of Saprolegniaceae (Oomycetes) inferred from LSU rDNA and ITS sequence comparisons. *Antonie van Leeuwenhoek*, **77**, 369–377.

Nicholls JA, Double MC, Rowell DM, Magrath RD (2000) The evolution of cooperative and pair breeding in thornbills *Acanthiza* (Pardalotidae). *Journal of Avian Biology*, **31**, 165–176.

Perotti MG, Basanta MD, Steciow MM, Sandoval-Sierra J V., Diéguez-Uribeondo J (2013) Early breeding protects anuran eggs from *Saprolegnia* infection. *Austral Ecology*, **38**, 672–679.

Petrisko JE, Pearl CA, Pilliod DS *et al.* (2008) Saprolegniaceae identified on amphibian eggs throughout the Pacific Northwest, USA, by internal transcribed spacer sequences and phylogenetic analysis. *Mycologia*, **100**, 171–180.

Riethmüller A, Weiß M, Oberwinkler F (2000) Phylogenetic studies of Saprolegniomycetidae and related groups based on nuclear large subunit ribosomal DNA sequences. *Canadian Journal of Botany*, **77**, 1790–1800.

Robideau GP, De Cock AWAM, Coffey MD *et al.* (2011) DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. *Molecular ecology resources*, **11**, 1002–1011.

Robinson J, Griffiths RA, Jeffries P (2003) Susceptibility of frog (*Rana temporaria*) and toad (*Bufo bufo*) eggs to invasion by *Saprolegnia*. *Amphibia-Reptilia*, **24**, 261–268.

Romansic JM, Diez KA, Higashi EM, Blaustein AR (2006) Effects of nitrate and the pathogenic water mold *Saprolegnia* on survival of amphibian larvae. *Diseases of aquatic organisms*, **68**, 235–243.

Romansic JM, Diez KA, Higashi EM, Johnson JE, Blaustein AR (2009) Effects of the pathogenic water mold *Saprolegnia ferax* on survival of amphibian larvae. *Diseases of aquatic organisms*, **83**, 187–193.

Romansic JM, Johnson PTJ, Searle CL *et al.* (2011) Individual and combined effects of multiple pathogens on Pacific treefrogs. *Oecologia*, **166**, 1029–1041.

Ruthig GR (2009) Water molds of the genera *Saprolegnia* and *Leptolegnia* are pathogenic to the North American frogs *Rana catesbeiana* and *Pseudacris crucifer*, respectively. *Diseases of aquatic organisms*, **84**, 173–178.

Sagvik J, Uller T, Olsson M (2008) A genetic component of resistance to fungal infection in frog embryos. *Proceeding of the Royal Society B*, **275**, 1393–1396.

Sandoval-Sierra JV, Martín MP, Diéguez-Uribeondo J (2014) Species identification in the genus *Saprolegnia* (Oomycetes): defining DNA-based molecular operational taxonomic units. *Fungal biology*, **118**, 559–78.

Stuart SN, Chanson JS, Cox NA *et al.* (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, **306**, 1783–1786.

Swan MJS, Cummins CP, Oldham RS (1994) Amphibians. In: *The Fresh Waters of Scotland* (eds Maitland PS, Boon PJ, McLusky DS), pp. 209–224. John Wiley & Sons, Ltd, Chichester, UK.

Tamura K, Peterson D, Peterson N *et al.* (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution*, **28**, 2731–2739.

Uller T, Sagvik J, Olsson M (2009) Pre-hatching exposure to water mold reduces size at metamorphosis in the moor frog. *Oecologia*, **160**, 9–14.

Urban MC, Lewis LA, Fučíková K, Cordone A (2014) Population of origin and environment interact to determine oomycete infections in spotted salamander populations. *Oikos*, 1–11.

Vanwest P (2006) *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. *Mycologist*, **20**, 99–104.

Table 1: Microbial species isolated per site, showing: the habitat from which samples were collected (Habitat); the total number of families isolated from each site (Total families); the number of species identified as *Saprolegnia* (*Saprolegnia sp.*); and the number of other eukaryotic microbial species identified (Other sp). Please see Table A1, Appendix, for further details of the species found.

Site	Habitat	Total families	*Saprolegnia* sp.	Other sp.
AU	Semi-rural woodland permanent pond	3	0	3
BI	Rural mountain temporary pool	3	0	3
BM	Rural woodland permanent marsh	5	2	5
BW	Rural farmland temporary pool	4	0	5
RE	Rural forestry temporary pool	4	0	4
CV	Rural forestry permanent pool	3	1	2
DM	Semi-rural country park permanent pond	3	1	2
GL	Rural roadside temporary pool	4	0	6
MU	Rural country park permanent loch	4	0	6
QP	Urban park permanent pond	8	2	7
Total		**24**	**4**	**33**

Table 2: Identification of species from each site where *Saprolegnia* was isolated, including: site of sample collection (Site), sample ID (Sample), the species identified via Genbank using the 28S and ITS regions, and sequence clusters assigned based on phylogeny using the 28S region (Cluster; Figure 2).

Site	Sample	Species identified using 28S	Cluster	Species identified using ITS
BM	BM1	*S. parasitica/litoralis*	C	NA
	BM2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	B	*S. diclina*
	BM3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	B	*S. diclina*
CV	CV1	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	B	*S. diclina*
	CV2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	B	*S. diclina*
	CV3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	B	*S. diclina*
	CV4	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	B	*S. diclina*
DM	DM1	*S. ferax/unispora*	A	*S ferax*
	DM2	*S. ferax/ unispora*	A	*S ferax*
	DM3	*S. ferax/unispora*	A	*S ferax*
	DM4	*S. ferax/ unispora*	A	*S ferax*
QP	QP1	*S. monilifera/ unispora/ torulosa/ terrestris*	D	NA

QP2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/hypogyna*	B	NA

NA: not available.

Figure 1: Sites of sample collection within central Scotland; triangles show sites where *Saprolegnia* species were present and circles show sites where *Saprolegnia* species were not isolated.

Figure 2: Bootstrap consensus tree with maximum-likelihood branch lengths from 28S rRNA sequences, showing reference sequences identified as *Saprolegnia* species in Genbank, alongside the sample sequences from this study. Bootstrap values above 60% are indicated but only those above 70% should be interpreted as resolved (Hillis & Bull 1993).

Figure 3: Boxplots of pH and altitude in relation to where *Saprolegnia* was present (1) or absent (0). Thick bars show the median trait value with interquartile ranges either side; whiskers show the range of values observed.

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APPENDIX

Table A1: All species identified at each site, including: site of sample collection (Site) and Sample ID in the form: site reference (e.g. R1), spawn clump reference per site (e.g. A), and egg number per clump (e.g. 1); alongside the species identified using Genbank (Species), the percentage of identical nucleotides between the sample and reference sequences within the alignment length (% match), and the Family of each species identified.

Site	Sample ID	Species	% match	Family
Auchinstarry	R3A2	*Dinemasporium pleurospora*	98	*Incertae sedis*
Auchinstarry	R3B1	*Microdochium phragmitis*	100	*Hyponectriaceae*
Auchinstarry	R3B3	*Hanseniaspora clermontiae*	100	*Saccharomycodaceae*
Banton Marsh	R2A1	*Didymella phacae*	100	*Pleosporomycetidae*
Banton Marsh	R2A3	*Trametes versicolor*	100	*Polyporaceae*
Banton Marsh	R2B2	*Phialemoniopsis curvata*	97	*Cephalothecaceae*
Banton Marsh	R2B3	*Didymella phacae*	100	*Pleosporomycetidae*
Banton Marsh	R2C1	*Didymella phacae*	100	*Pleosporomycetidae*

Banton Marsh	R2C2	*Curreya pityophila*	100	*Pleosporomycetidae*
Banton Marsh	R2C3	*Curreya pityophila*	100	*Pleosporomycetidae*
Banton Marsh	R2D1	*S. parasitica/litoralis*	98	*Saprolegniaceae*
Banton Marsh	R2D2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Banton Marsh	R2D3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Banton Marsh	R2E1	*Curreya pityophila*	100	*Pleosporomycetidae*
Banton Marsh	R2E3	*Monographella lycopodina*	100	*Amphisphaeriaceae*
Banton Wood	R1A1	*Microdochium phragmitis*	99	*Incertae sedis*
Banton Wood	R1A2	*Microdochium phragmitis*	99	*Hyponectriaceae*
Banton Wood	R1B1	*Microdochium phragmitis*	99	*Hyponectriaceae*
Banton Wood	R1B2	*Guehomyces pullulans*	80	*Cyfstofilobasidiaceae*
Banton Wood	R1B3	*Guehomyces pullulans*	98	*Cyfstofilobasidiaceae*
Banton Wood	R1C1	*Microdochium phragmitis*	84	*Hyponectriaceae*
Banton Wood	R1D1	*Monographella lycopodina*	100	*Amphisphaeriaceae*
Banton Wood	R1D3	*Phoma herbarum*	99	*Incertae sedis*
Banton Wood	R1E1	*Neottiosporina paspali*	99	*Incertae sedis*
Banton Wood	R1E3	*Microdochium phragmitis*	99	*Hyponectriaceae*

Ben Ime	R4B1	*Trichoderma viride*	82	*Hypocreaceae*
Ben Ime	R4D3	*Varicosporium delicatum*	99	*Helotiaceae*
Ben Ime	R4E2	*Mortierella fimbricystis*	95	*Mortierellaceae*
Carron Reservoir	R7B1	*Bionectria ochroleuca*	100	*Bionectriaceae*
Carron Reservoir	R7B2	*Hypholoma acutum*	99	*Strophariaceae*
Carron Reservoir	R7D1	*Mortierella elongata*	99	*Mortierellaceae*
Carron Reservoir	R7E1	*Microdochium phragmitis*	98	*Hyponectriaceae*
Carron Valley	R8B3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Carron Valley	R8C1	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Carron Valley	R8C2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Carron Valley	R8C3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	99	*Saprolegniaceae*
Carron Valley	R8D1	*Mortierella elongata*	99	*Mortierellaceae*
Carron Valley	R8D3	*Ceratobasidium cornigerum*	98	*Ceratobasidiaceae*
Carron Valley	R8E1	*Mortierella elongata*	99	*Mortierellaceae*
Darnley Mill	U2A2	*S. ferax/ unispora*	100	*Saprolegniaceae*
Darnley Mill	U2B3	*S. ferax/ unispora*	100	*Saprolegniaceae*
Darnley Mill	U2C1	*S. ferax/unispora*	100	*Saprolegniaceae*

Darnley Mill	U2C2	*Westerdykella multispora*	100	*Sporormiaceae*
Darnley Mill	U2D3	*Paraphoma chrysanthemicola*	99	*Incertae sedis*
Darnley Mill	U2E2	*S. ferax/unispora*	100	*Saprolegniaceae*
Glen Luss	R5A1	*Mazzantia angelicae*	99	*Diaporthaceae*
Glen Luss	R5A2	*Phaeocytostroma ambiguum*	99	*Incertae sedis*
Glen Luss	R5B1	*Phoma complanata*	100	*Incertae sedis*
Glen Luss	R5B3	*Hymenoscyphus tetracladius*	99	*Helotiaceae*
Glen Luss	R5C1	*Trametes versicolor*	99	*Polyporaceae*
Glen Luss	R5C3	*Didymella phacae*	99	*Pleosporomycetidae*
Glen Luss	R5E1	*Didymella phacae*	99	*Pleosporomycetidae*
Glen Luss	R5E2	*Didymella phacae*	99	*Pleosporomycetidae*
Mugdock	R6A2	*Pseudeurotium hygrophilum*	99	*Pseudeurotiaceae*
Mugdock	R6B1	*Pseudeurotium zonatum*	99	*Pseudeurotiaceae*
Mugdock	R6B3A	*Varicosporium scoparium*	96	*Helotiaceae*
Mugdock	R6B3B	*Sarcoleotia turficola*	96	*Geoglossaceaee*
Mugdock	R6D1	*Didymella phacae*	99	*Pleosporomycetidae*
Mugdock	R6D2	*Globisporangium paddicum*	99	*Pythiaceae*

Mugdock	R6D3	*Plectosphaerella plurivora*	100	*Plectosphaerellaceae*
Queens Park	U1A1	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/hypogyna*	99	*Saprolegniaceae*
Queens Park	U1A3	*Trametes versicolor*	99	*Polyporaceae*
Queens Park	U1C1A	*Trichoderma viride*	99	*Hypocreaceae*
Queens Park	U1C1B	*Boeremia exigua*	100	*Pleosporomycetidae*
Queens Park	U1C2A	*Penicillium solitum*	100	*Trichocomaceae*
Queens Park	U1C2B	*Westerdykella multispora*	100	*Sporormiaceae*
Queens Park	U1C3A	*Mucor hiemalis*	100	*Mucoraceae*
Queens Park	U1C3B	*Penicillium solitum*	100	*Trichocomaceae*
Queens Park	U1D1	*Mucor hiemalis*	99	*Mucoraceae*
Queens Park	U1D1	*S. monilifera/ unispora/ torulosa/ terrestris*	99	*Saprolegniaceae*
Queens Park	U1E1	*Trametes versicolor*	99	*Polyporaceae*
Queens Park	U1E2	*Neobulgaria pura*	96	*Lachnaceae*

Table A2: Identification of species from each site where *Saprolegnia* was isolated using the 28S and ITS region, including: site of sample collection (Site), sample ID (Sample), the species identified via Genbank, the percentage of identical nucleotides between the sample and reference sequences within the alignment length (% match), and the percentage of the sample sequence covered by the reference sequence (% coverage).

Site	Sample	Species identified using 28S	Species identified using ITS	% match 28S	% coverage 28S	% match ITS	% coverage ITS
BM	BM1	*S. parasitica/litoralis*	NA	98	100/100	NA	NA
	BM2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	*S. diclina*	99	100/90/100/100/ 100/100/93	100	99
	BM3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	*S. diclina*	99	100/90/100/100/ 100/100/93	100	100
CV	CV1	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	*S. diclina*	99	100/90/100/100/ 100/100/93	100	100
	CV2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	*S. diclina*	99	100/90/100/100/ 100/100/93	100	100
	CV3	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	*S. diclina*	99	100/90/100/100/ 100/100/93	100	100
	CV4	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/ hypogyna*	*S. diclina*	99	100/90/100/100/ 100/100/93	100	99
DM	DM1	*S. ferax/unispora*	*S ferax*	100	100/100	100	99
	DM2	*S. ferax/ unispora*	*S ferax*	100	100/100	97	97
	DM3	*S. ferax/unispora*	*S ferax*	100	100/100	100	99
	DM4	*S. ferax/ unispora*	*S ferax*	100	100/100	97	97
QP	QP1	*S. monilifera/ unispora/ torulosa/ terrestris*	NA	99	100/100/99/99	NA	NA

QP2	*S. diclina/ ferax/ unispora/ mixta/ parasitica/ delica/hypogyna*	NA	99	100/90/100/100/ 100/100/93	NA	NA

Table A3: Environmental parameter measurements taken at sample collection, including the water parameters: conductivity, total dissolved solids, pH and temperature; and the geographical parameter: altitude.

Site	Conductivity (µS)	Dissolved solids (ppm)	pH	Temperature (°C)	Altitude (m)
AU	111	97	6.3	12.1	19
BI	189	107	6.4	21	93
BM	102	50	6.5	10.4	72
BW	333	233	5.7	7.7	179
CV	56	29	6.1	12.1	228
DM	250	133	6.4	13.1	149
GL	45	23	5.8	15.1	50
MU	149	78	6.1	12.9	51
QP	127	65	6.4	12.5	163
RE	75	39	6.3	11.7	53