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# Culture media and temperature influence on growth and sexual reproduction of the fish pathogens *Achlya racemosa* and *Saprolegnia ferax*

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With 2 figures and 2 tables

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**Abstract**: Two fungal parasites isolated from the fish *Odontesthes bonariensis* in Argentina were identified as *Achlya racemosa* and *Saprolegnia ferax* based on morphology and ITS rDNA sequence comparison. Studies were conducted to determine the effect of culture temperature and culture medium on growth rate and stimulation of sexual reproduction. Both isolates showed an increased rate of vegetative growth with increasing temperature; the growth rate of *Saprolegnia ferax* was higher than that of *Achlya racemosa*. Culture medium did not significantly affect growth rate of either species at the same temperature. However, differences attributable to culture medium in the formation of sexual structures were detected.

**Keywords**: ITS region rDNA, molecular characterization, fish parasites, growth rate, Saprolegniales, sexual reproduction.

## Introduction

Saprolegniales, also known as water molds, are among the most widespread aquatic fungi occurring in fresh water, estuaries and moist soils. Most species are saprotrophic; however, some of them are capable of causing disease in fish and their eggs, both under

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natural conditions and in culture systems (Pickering et al. 1979, Hatai et al. 1990, Hatai & Hoshina 1992, Kitancharoen et al. 1996, Dieguez-Uribeondo et al. 1996).

Although the presence of these fungi on the host is easily recognized by their cottony aspect, correct species-level identification is usually difficult since it depends mainly on the observation, description and measurement of sexual and asexual morphological characters (Seymour 1970, Dick 2001, Johnson et al. 2002). Due to the fact that the formation of such structures is slow or scarce in some species, several authors have focused on the search for other differential features that could allow faster identification. Thus, morphological characteristics such as type of germination (Willoughby et al. 1983), spore ornamentation (Pickering et al. 1979), or physiological characteristics such as peptone hydrolysis, carbohydrate assimilation profiles, or urea hydrolysis (Wolf 1937, Yuasa & Hatai 1996) are currently used as additional parameters for classification and identification. Over the recent years molecular analysis has come to the rescue of taxonomists and has become a useful tool to supplement the morphological differences between species (de Cock & Levesque 2004). Comparative studies of the internal transcribed spacer (ITS) regions of the ribosomal RNA genes (rDNA) have become very important tools in fungal taxonomy as these regions evolve fast enough so as to distinguish different species within a genus (White et al. 1990, Lee & Taylor 1992). Amplification of the ribosomal genes is used for the genetic identification of many organisms because they comprise both highly conserved sequences during evolution and highly variable sequences among species and even within species. This comparison has already been used for the identification of new species (de Cock & Levesque 2004, Paul 2006, Paul & Bala 2008, Paul & Steciow 2004–2008, El Androusse et al. 2006). The ITS sequence data can provide valuable information on "new" or undescribed taxa, as sequence diversity will support the identification of new species (Cooke et al. 1999).

This work presents a study on the ITS region of the rDNA and the effects of temperature and culture medium on the growth rate and formation of taxonomically significant sexual characters in two genera of the family *Saprolegniaceae*, both associated with parasitic activity on silversides *Odontesthes bonariensis* (Cuvier & Valenciennes), Atherinopsidae (Osteichthyes).

It is necessary to enlarge our knowledge concerning the use of nutrients by parasitic species, in order to produce culture media with precise chemical composition that may not only facilitate their rapid isolation and identification, but also support both their vegetative growth and the development of sexual organs and dispersal structures.

## Materials and methods

OOMYCETEOUS MATERIALS: Eggs of silverside were provided by the Chascomús Fisheries Experimental Station located in Chascomús (Buenos Aires Province, Argentina) and taken to the Laboratory of Pathology of Aquatic Organisms, where they were incubated. During incubation infected eggs covered by white cottony fungal mycelium were detected. Two months later, a juvenile silverside specimen was found to have a perianal lesion covered by abundant mycelium. Two strains used in this work, isolated from eggs and one juvenile individual of silverside *Odontesthes bonariensis,* were identified as *Achlya racemosa* Hildebr. (LPSC 1019) and *Saprolegnia ferax* (Gruith.) Thuret (LPSC1020) and deposited in the culture collection of Instituto de Botánica Spegazzini.

Species-level identification was initially carried out on the basis of morphological characters, following the criteria of Seymour (1970) and Johnson et al. (2002). Measurements such as diameter of fungal colonies, diameter of oogonia, number of oospores per oogonia, morphology and diameter of oospores, and other morphological characteristics of antherdial branches and oogonia, were determined from 50 counts of each of three replicates using an Olympus BX 40 microscope equipped with phase contrast optics. A detailed morphological characterization and the demonstration of pathogenicity of the species have been previously reported by Pacheco Marino et al. (2009). Molecular analysis was done by the extraction of DNA and the amplification and sequencing of the ITS region of their ribosomal DNA.

DNA EXTRACTION, PCR AMPLIFICATION & SEQUENCING: The oomycetes were grown in potato dextrose broth. DNA was extracted from the oomycete mycelium using the method described earlier (Paul & Steciow 2004, 2008, El Androusse et al. 2006) in which DNA was purified from mycelia using the DNAEasy Plant Mini kit (Qiagen, Basel, Switzerland) according to the manufacturer's specifications. Quality was checked by visualization under UV light following electrophoretic separation with a molecular mass standard in 1% agarose (Biofinex) gel in 1× TBE, subjected to 100 V for 1 h and stained with ethidium bromide (0.5 mg/ml). Concentrations were assayed in a S2100 Diode Array spectrophotometer (WPA Biowave, Cambridge, UK). The primers were provided by Genome express (Meylan, France).

ITS amplifications of the oomycetes samples were carried out using previously described universal primers ITS1 and ITS4 (White et al. 1990, Cooke et al. 1999). The reaction mixture contained 1× PCR buffer (75 mM Tris-HCl (pH 9.0), 50 mM KCl, 20 mM $(NH_4)_2 SO_4$), 0.1 mM dNTPs, 0.25 µM of each primer, 1.5 mM $MgCl_2$, 1 µl of Taq Polymerase (Biotools, Spain) and 1 µl of DNA in a total volume of 50 µl. Amplifications were carried out in a Master Gradient thermocycler (Techne, Progene, France) according to the following amplification program: an initial denaturation step at 95°C for 2 min followed by 30 cycles including denaturation at 95°C for 20 s, annealing for 25 s at 55°C and extension for 50 s at 72°C. Amplification was finished by a final extension step for 10 min at 72°C (Cooke et al. 1999). PCR products were separated in 1% agarose (Biofinex, Switzerland) gels in 1x TBE subjected to 100 V for 1 h, stained with ethidium bromide (0.5 mg/l) and visualised under UV light. Amplicons were purified using a Minelute PCR Purification Kit (Qiagen) according to manufacturer's specifications. Quantity and quality were checked as described above for DNA extraction. Amplicons were sequenced directly in both sense and antisense directions. All oomycete samples were sequenced twice and a consensus sequence was created from the duplicates. The sequencing was performed by Genome express (Meylan, France). DNA sequences were deposited in GenBank.

EFFECT OF CULTURE MEDIUM AND TEMPERATURE: Subcultures were made from the isolated pure strains on 90 × 20 mm Petri dishes containing 20 ml of corn meal (CM) agar and 1 ml of antibiotic solution (1 g streptomycin sulphate and 0.5 g chloramphenicol in 200 ml of sterile distilled water). After 48 hours, inocula (approx. 2 mm in diameter) were taken from the active growth margin and placed in the center of a Petri dish with 20 ml of agar. The inoculum was seeded in triplicate on four culture media: Emerson's YpSs (Yeast Extract-Soluble Starch) agar (Stevens 1974), Glucose-Glutamate agar (GGl) (Seymour 1970), corn meal agar (CM) (Fuller & Jaworsky 1987) and Glucose-Yeast extract (GY) agar (Hatai & Hoshina 1992). The inoculated plates were placed in incubation chambers at 5, 15 and 25°C, and colony diameter was measured daily using a Vernier caliper (1 mm accuracy). Recording of colony diameter was stopped when the Petri dish was full. The formation of sexual and asexual reproductive structures was also recorded during the experiment at twenty four hours intervals.

STATISTICAL ANALYSES: Growth rate was calculated by means of linear regression using Microsoft Excel 2002 to obtain the linear equation, Pearson's correlation coefficient and the coefficient of determination $R^2$. Then the variance was analyzed by means of a two-factor ANOVA to evaluate the existence of significant differences between growth rates as well to analyze the interaction between Temperature and Culture medium, using the software program XL Stat 7.5. Finally, the optimal temperature and culture medium for development were determined through Dunnet's multiple comparison method, using the temperature (15°C) and culture medium (CM) as control standards.

## Results

### Sequence analysis of *Achlya racemosa* and *Saprolegnia ferax*

***Achlya racemosa*** LPS 1019: The ITS2 region of this oomycete (GenBank accession number EU551150) was composed of 324 bases, it showed 100% identity with the sequence deposited under GenBank accession number AF218158 which confirmed our morphological identification. The sequence of our isolate was also very close to that of *A. colorata* (AF18159) having 99% similarity, *A. radiosa* (AF218160) 98%, and also *Saprolegnia turfosa* (AB219397).

***Saprolegnia ferax*** LPS1020: The ITS region (GQ119935) was composed of 654 bases; a BLAST search evidenced 100% similarity with *Saprolegnia ferax* isolated from amphibian eggs (EU124763).

### Effect of temperature on vegetative growth rate

As shown in Figures 1 A and B, in both species growth was related to incubation temperature. The growth rates for each condition are shown in Table 1.

#### a) *Achlya racemosa*

At 25°C *A. racemosa* reached maximum colony diameter after 6 days on CM, 7 days on GY and 8 days on YpSs and GGl. At 15°C, maximum colony diameter was reached after 9 days on CM and GY, 10 days on YpSs and 11 on GGl. Finally, at 5°C, maximum growth was attained after 24 days on CM, 27 days on YpSs, 29 days on GY and 34 days on GGl.

As shown in Table 1, the growth rate at 25°C was higher on CM, as statistically tested; in addition, no statistically significant differences were found between CM and GY or between GGl and YpSs ($p < 0.001$), but there were significant differences between CM and the other culture media. At 15°C, CM was also the medium with the highest growth rate, but significant differences were only found between GY and GGl ($p = 0.023$), YpSs and CM ($p = 0.038$) and CM and GY ($p = 0.009$). Tukey's analysis of between-groups differences (HSD) indicated that for 95.0% confidence interval, no significant differences in growth rate existed between YpSs at 25°C and GY at 15°C; YpSs at 25°C and CM at 15°C; GGl at 25°C and GY at 15°C; GGl at 25°C and CM at 15°C ($p > 0.05$). At 5°C, no statistically significant differences were found between growth rates for the different culture media.

#### b) *Saprolegnia ferax*

Figure 1 shows the growth of *Saprolegnia ferax* at different experimental temperatures. This species reached maximum colony diameter after 4 days on CM agar, and 5 days on GY, YpSs and GGl at 25°C. At 15°C, maximum colony diameter was reached after 6 days on CM agar, GY and YpSs, and 7 days on GGl. At 5°C, maximum colony diameter was reached after 14 days on YpSs, 16 days on GY and 22 days on CM and GGl.

As shown in Table 1, the growth rate was higher on CM, but Tukey's analysis of group differences (HSD, IC = 95%) showed that at this temperature, significant

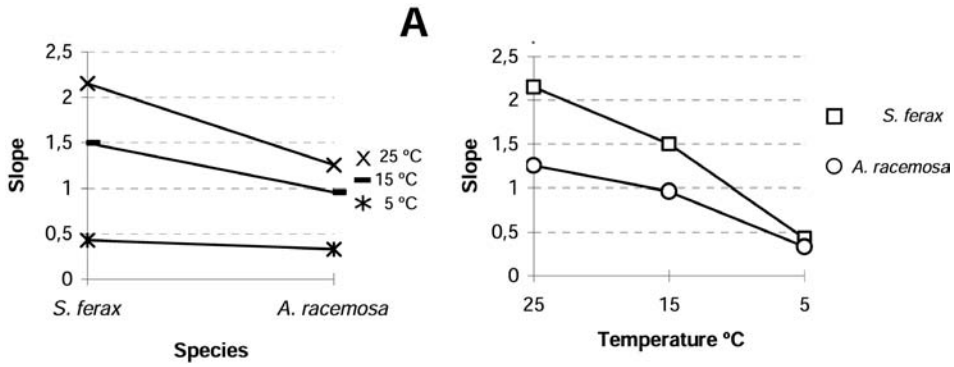


Fig. 1. A–B. Interaction effect of factors Temperature and Species on the growth rate of *Achlya racemosa* and *Saprolegnia ferax.* Y = growth rate.

differences in growth rate only existed between GGl and GY ($p = 0.006$), YpSs and CM ($p = 0.015$) and lastly between GGl and CM ($p = 0.046$). At 15°C, CM was also the medium with the highest growth rate, but the different pairs were only GY and GGl ($p = 0.001$), and CM and GGl ($p = 0.006$). As in the case of *Achlya racemosa*, at 5°C there were no statistically significant differences in the growth rates in different culture media. Apparently, temperature would have greater influence on the growth rate of this species compared to *Achlya racemosa*.

Table 1 shows that the growth rates of *Saprolegnia ferax* were higher than those of *Achlya racemosa*. An ANOVA confirmed that the difference was statistically significant ($0.05\ p < 0.01$). Furthermore, this difference tends to diminish at decreasing temperature.

As shown in Figure 1 A–B, the growth rate was the highest at 25°C for both species. The ANOVA showed statistically significant differences in growth rates between the temperatures tested (IC 95%, $p < 0.001$), and a subsequent Dunnet's multiple comparisons test showed that the most rapid growth occurred at 25°C (IC 95%, $p < 0.001$).

Figure 2 shows the growth rates for different culture media in agreement with the observations. An ANOVA test revealed significant differences in growth rate between the different culture media except between CM and GY; Dunnet's a posteriori analysis indicated that the growth rate was higher for CM.

As shown in Figures 2 A and B, the effect of culture medium was influenced by incubation temperature; these differences were less conspicuous at 5°C. Figure 6A also shows that the effect of culture medium on growth rate was similar for both species, and that highest growth rate occured on GY and CM.

**Effect of temperature on the development of taxonomically relevant characters**

As shown in Table 2, the fastest formation of oogonia of *Saprolegnia ferax* occurred on GGl, at 15 and 25°C; whereas at 5°C oogonia formation was fastest on GY; this

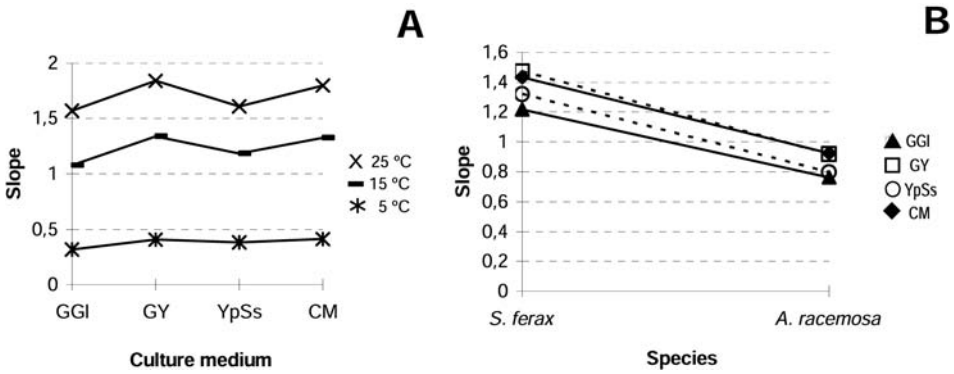


Fig. 2. A–B. Interaction effect of factors Culture medium and Species on the growth rate of *Achlya racemosa* and *Saprolegnia ferax.* Y = growth rate.

species did not form oogonia on CM at any temperature. On the other hand, *Achlya racemosa* formed oogonia faster on GY and CM at 15 and 25°C, whereas the formation of oogonia at 5°C was fastest on CM.

## Discussion

One of the first observations about the nutritional physiology of aquatic fungi parasitic on fishes was made by Robins (1853), who proposed that the animals secreted substances that provided nutrients for their development and growth. However, many years elapsed until one of the pioneers in the development of methods for obtaining pure cultures demonstrated the possibility of isolating and maintaining cultures of Saprolegniales isolated from fishes (Johnson et al. 2002). Since then, several authors have focused their efforts on the formulation of culture media that allow both the vegetative growth of these organisms and the production of sporangia or buds. Thus, Kanouse (1932) was the first to induce sexual reproduction in cultured *Saprolegnia parasitica*, a species that had been considered as a producer of sterile mycelium up to that moment.

Given the taxonomical importance of the development of morphological characters that are representative of each species, our goal was to determine the most adequate medium formulation and culture conditions to facilitate the development and fast characterization of these pathogenic species.

Our results showed that the growth rate of *S. ferax* was higher than that of *Achlya racemosa*, but both species showed the same trend toward faster growth at increasing incubation temperature. We also found that both species were capable of growing at 5°C, but the growth rates were considerably higher at 15 and 25°C. These results agreed with Nolan's (1976) research on *Saprolegnia ferax* isolated from the gut of the blackfly *Simulium vittatum*; this author found an optimum growth temperature

Table 1. Values of slope, Pearson correlation coefficient and Coefficient of Determination $R^2$, and growth rate (cm/day) for *Achlya racemosa* and *Saprolegnia ferax*, grown on GGl, GY, YpSs and CM and incubated at 25, 15 and 5°C.

| | Temperature | Culture medium | Slope | Pearson Coefficient | Coefficient of determination $R^2$ | Growth rate (cm/day) |
|---|---|---|---|---|---|---|
| *Saprolegnia ferax* | 25°C | GGl | 2.006 | 1.000 | 0.975 | 1.80 |
| | | GY | 2.316 | 0.975 | 0.906 | 1.80 |
| | | YpSs | 2.034 | 0.977 | 0.949 | 1.80 |
| | | CM | 2.254 | 1.000 | 0.814 | 2.25 |
| | 15°C | GGl | 1.347 | 0.993 | 0.934 | 1.48 |
| | | GY | 1.697 | 0.999 | 0.974 | 1.79 |
| | | YpSs | 1.471 | 0.997 | 0.981 | 1.50 |
| | | CM | 1.626 | 0.997 | 0.990 | 1.80 |
| | 5°C | GGl | 0.362 | 0.982 | 0.858 | 0.27 |
| | | GY | 0.500 | 0.988 | 0.871 | 0.53 |
| | | YpSs | 0.453 | 0.992 | 0.886 | 0.49 |
| | | CM | 0.463 | 0.991 | 0.960 | 0.47 |
| *Achlya racemosa* | 25°C | GGl | 1.132 | 0.997 | 0.992 | 1.13 |
| | | GY | 1.347 | 0.999 | 0.997 | 1.29 |
| | | YpSs | 1.235 | 0.996 | 0.986 | 1.13 |
| | | CM | 1.369 | 0.998 | 0.917 | 1.50 |
| | 15°C | GGl | 0.864 | 0.998 | 0.995 | 0.82 |
| | | GY | 1.038 | 0.997 | 0.990 | 1.00 |
| | | YpSs | 0.895 | 0.999 | 0.996 | 0.90 |
| | | CM | 1.057 | 0.989 | 0.973 | 1.13 |
| | 5°C | GGl | 0.281 | 0.991 | 0.974 | 0.25 |
| | | GY | 0.346 | 0.994 | 0.971 | 0.29 |
| | | YpSs | 0.319 | 0.999 | 0.981 | 0.29 |
| | | CM | 0.374 | 0.999 | 0.977 | 0.33 |

range of 15–26°C. Studies on other Saprolegniales species, such as those by Rakmanee et al. (2004) in *Saprolegnia diclina* and *Achlya ambisexuales,* parasitic on *Cyprinus carpio* L.*,* indicated an optimal temperature for both species of 25°C, though growth occurred within a temperature range of 10–30°C and 15–30°C, respectively. In turn, Koeypudsa et al. (2005) studied the growth of several *Saprolegnia* sp. strains collected from diseased fishes in Norway and Chile, obtaining the maximum growth at 25°C wich was inhibited at temperatures higher than 30°C.

Kitancharoen et al. (1996) found that several isolates of *Saprolegnia* showed similar growth at 10 and 20°C. However, at 30°C the isolates from visceral mycosis showed rapid growth whereas the growth of isolates from external mycosis was slow to inexistent, thus indicating a relationship between temperature tolerance and provenance of the isolate. These studies have shown that aquatic fungi are able to survive in environments with highly diverse temperatures without the latter affecting their capacity for growth, reproduction and dissemination. This characteristic favors their infective capacity on fish affected by stress due to changes in environmental temperature (Olah & Farkas 1978, Kitancharoen et al. 1996, Rakmanee et al. 2004).

Table 2. Formation of oogonia in *Achlya racemosa* and *Saprolegnia ferax* grown on GGl, GY, YpSs and CM and incubated at 25, 15 and 5°C.

| | | Formation of oogonia (days) | |
|---|---|---|---|
| Temperature | Culture medium | *Saprolegnia ferax* | *Achlya racemosa* |
| 25°C | GGl | 4 | 6 |
| | GY | 4 | 5 |
| | YpSs | – | – |
| | CM | – | 5 |
| 15°C | GGl | 5 | 6 |
| | GY | 9 | 4 |
| | YpSs | 8 | 6 |
| | CM | – | 4 |
| 5°C | GGl | 20 | 18 |
| | GY | 16 | 20 |
| | YpSs | 18 | 18 |
| | CM | – | 16 |

The differences between the growth rates of both species are noteworthy, since several authors have suggested that these would be related to host specificity and could be useful in terms of classification, contributing to a more rapid identification of species and subgroups within species, evidencing differences between different strains (Hatai et al. 1990, Dieguez-Uribeondo et al. 1996, Kitancharoen et al. 1996). In this respect, Wood et al. (1988) observed growth differences at 25°C between *S. diclina* and *S. parasitica*, parasites of salmon. Hatai & Hoshina (1992) noted that the growth rates at 30°C could be used for rapid identification of *Saprolegnia* sp. Husein & Hatai (1999) classified *Saprolegnia* sp. on the basis of its growth rate at temperatures ranging between 3–33°C, while Wolf (1937) found that unlike *Achlya bisexualis*, *Saprolegnia ferax* was able to grow at 2°C.

The statistical analysis led us to conclude that temperature is the only factor that significantly affects the vegetative growth of *Achlya racemosa* and *Saprolegnia ferax*, since no significant differences were found in the vegetative growth of mycelia on different culture media at the same temperature.

According to our results, GGl and GY would be the most adequate culture media to obtain rapid formation of oogonia in *Saprolegnia ferax* at 15 and 25°C. This rapid formation of *S. ferax* oogonia on GGL and GY could be due to phosphates present in the culture medium that would stimulate its reproductive capacity, as observed in other species such as *S. mixta* and *S. hypogyna* (Klebs 1899, Kaufman 1908). These findings would also explain the absence of sexual reproduction in the tests made on media with lower phosphate concentrations, such as CM agar (Johnson et al. 2002) and potato dextrose agar (Wolf 1937).

Unlike *Saprolegnia ferax*, *Achlya racemosa* formed oogonia on GY and CM at 15 and 25°C, whereas at 5°C the formation of oogonia was faster on CM agar.

Thus, there are apparently marked differences between both species with regards to their use of nutrients for the development of sexual characters. Our experimental evidence agrees with the theory of specificity proposed by Klebs (1899) and later demonstrated by Kanouse (1932) for *Saprolegnia parasitica*, which states that each species would have a characteristic capacity of nutrient assimilation for the purpose of forming sexual structures.

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