Accepted Manuscript

Title: Detection of 'long-haired' *Saprolegnia* (*S. parasitica*) isolates using monoclonal antibodies

Authors: Juan M. Fregeneda-Grandes, Fausto Rodríguez-Cadenas, M. Teresa Carbajal-González, J. Miguel Aller-Gancedo

PII: S0953-7562(07)00090-1
DOI: 10.1016/j.mycres.2007.04.005
Reference: MYCRES 261

To appear in: *Mycological Research*

Received Date: 17 July 2006
Revised Date: 14 December 2006
Accepted Date: 17 April 2007

Please cite this article as: Fregeneda-Grandes J.M., Rodríguez-Cadenas F., Carbajal-González M.T., Aller-Gancedo J.M. Detection of 'long-haired' *Saprolegnia* (*S. parasitica*) isolates using monoclonal antibodies, Mycological Research (2007), doi: 10.1016/j.mycres.2007.04.005

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Detection of 'long-haired' *Saprolegnia* (*S. parasitica*) isolates using monoclonal antibodies

Juan M. FREGENEDA-GRANDES, Fausto RODRÍGUEZ-CADENAS, M. Teresa CARBAJAL-GONZÁLEZ, J. Miguel ALLER-GANCEDO*

Departamento de Sanidad Animal, Facultad de Veterinaria, Universidad de León, E-24071 León, Spain

***Corresponding author**. Tel.: +34 987 291 303.

E-mail address: dsajmag@unileon.es

Running title: Monoclonal antibodies recognizing *Saprolegnia parasitica*

The ability of five monoclonal antibodies (Mabs) raised against a pathogenic *Saprolegnia parasitica* isolate from brown trout to detect and differentiate between isolates with bundles of long hairs (*S. parasitica*) and other *Saprolegnia* species was determined by means of an indirect immunofluorescence assay. Four of the Mabs used recognized some of the long-haired *S. parasitica* isolates but also cross-reacted with other *Saprolegnia* species without bundles of hairs and with *Achlya* sp. The other Mab (named 18A6) was able to differentiate between the asexual and most of the sexual isolates in the group of long-haired *S. parasitica* isolates, but did not recognize *Achlya* sp. or the *Saprolegnia* species without bundles of hairs, with the exception of *S. hypogyna*. These results indicate that isolates with bundles of long hairs are closely related with other members of genus *Saprolegnia* and share several antigens. However, Mab 18A6 seems to recognize an epitope that is expressed mainly in the asexual isolates in the long-haired *S. parasitica* isolates.

ACCEPTED MANUSCRIPT

ARTICLE IN PRESS

Keywords: *Oomycetes*, saprolegniosis, taxonomy, immunofluorescence, brown trout.

Introduction

Infections caused by *Saprolegnia* spp. (*Saprolegniaceae*, *Saprolegniales*, *Oomycetes*) are important freshwater fish diseases, which often affects wild and farmed salmonids in freshwater environments (Pickering & Willoughby 1982, Noga 1993, Bruno & Wood 1999). In salmonid fish farms, the disease affects mainly broodfish and incubating eggs, causing major financial losses. In rivers and lakes, saprolegniosis can reduce the natural salmonid populations, as has occurred amongst wild brown trout *Salmo trutta* in several rivers in the Province of Leon in Spain (Aller Gancedo & Fernández Díez 1987). Taxonomic identification of *Saprolegnia* species is based mainly on the morphology of the sexual reproductive structures produced in culture (Seymour 1970, Johnson *et al.* 2002: *Biology and Systematics of the Saprolegniaceae*, available at: http://www.ilumina-dlib.org and http://www.uncw.edu/people/padgett). However, such identification is sometimes difficult or not possible, especially with salmonid pathogenic isolates, which frequently do not produce sexual structures under laboratory conditions, and when they do develop, they appear to be very similar to those observed in the saprophytic species *S. diclina*. Several studies (see Beakes *et al.* 1994 for summaries of these) have provided evidence that isolates causing salmonid fish lesions all grouped into a coherent, separate cluster based on cyst coat morphology (bundles of long, hooked hairs in secondary cysts), germ tube growth (indirect germination in low nutrient medium), and esterase isozyme patterns (one to five fast-migrating bands). Theoretically, these allow a distinction to be made between parasitic and saprophytic isolates, like *S. diclina*. Nonetheless, these characteristics have also been observed in isolates obtained from water or other freshwater fish and crayfish, and are not always directly related to pathogenicity. Hence, in previous studies we have described two different morphotype groups of long-haired *Saprolegnia* isolates obtained from brown

ACCEPTED MANUSCRIPT

trout and from river water in Spain. The results indicated that most isolates from water belonged to morphotype group I (with a greater number and longer bundles of hairs) and had low or no pathogenicity, despite their cyst coat ornamentation. In contrast, the more pathogenic isolates belonged to morphotype group II, with less numerous and shorter bundles of hairs, usually being isolated from salmonids with saprolegniosis (Fregeneda-Grandes *et al.* 2000, 2001). In a recent study, we investigated the phylogenetic relationship among isolates of the *S. diclina–S. parasitica* complex on the basis of ITS regions of nu-rDNA, in which we found that pathogenic isolates grouped together in a phylogenetically distant clade from *S. diclina,* and it is suggested that all isolates of this clade be termed *S. parasitica* (Diéguez-Uribeondo *et al.* 2007).

In this context, the development of monoclonal antibodies (Mabs) could particularly useful in differentiating between pathogenic and non-pathogenic isolates or species within the *S. diclina–S. parasitica* complex and other *Saprolegnia* species. A number of studies have demonstrated the usefulness of Mabs for examining the biology, taxonomy, and pathogenesis of other major *Oomycete* pathogens, such as *Phytophthora cinnamomi* (Hardham *et al.* 1986, Gabor *et al.* 1993), *Pythium aphanidermatum* (Estrada-García *et al.* 1989), or *Aphanomyces invadans* (Miles *et al.* 2003). However, it would appear that very few studies using Mabs raised against *Saprolegnia* species pathogenic to fish have been published (Burr & Beakes 1994, Beakes *et al.* 1995, Bullis *et al.* 1996).

The aim of the present work was to determine whether Mabs raised against a salmonid-pathogenic *S. parasitica* isolate could detect and differentiate between pathogenic and non-pathogenic isolates from within the *S. diclina–S. parasitica* complex and other *Saprolegnia* species.

ACCEPTED MANUSCRIPT

Materials and methods

ARTICLE IN PRESS

Fungal isolates

A total of 48 *Saprolegniaceae* isolates (Table 1) were used in the present study. They were all obtained in Northwest Spain, mainly from wild brown trout suffering from saprolegniosis or from the water from various rivers. Thirty-five of these isolates, now renamed *Saprolegnia parasitica* in line with Diéguez-Uribeondo *et al.* 2007, had bundles of long hairs in the secondary cysts, considered to be one of the major characteristics of salmonid-pathogenic strains (Fig. 1). Seventeen of them developed sexual structures at 7 °C, but not at 20 °C, with or without antheridial invested oogonia (formerly *S. diclina* Type 1). The remaining 18 isolates were asexual (formerly *Saprolegnia* spp.). Another 12 *Saprolegnia* isolates without bundles of hairs [five *S. diclina* (Fig. 1), two *S. australis*, two *S. ferax*, two *S. hypogyna*, and one *S. terrestris*] and one isolate of *Achlya* sp. were also used. Stock cultures of these isolates were maintained at a temperature of between 4 and 7 °C in glass bottles with sterilized distilled water and a single hemp seed as a culture medium (Aller-Gancedo *et al.* 1999). All the *Saprolegniaceae* isolates used in the present study have been permanently preserved at the fungus culture collection belonging to the Department of Animal Health, Faculty of Veterinary, University of León.

Preparation of antigens

Hyphal antigens were prepared as described by Bullis *et al.* (1990). Fungal isolates were cultivated in flasks containing 50 ml of GY broth (1 % glucose and 0.25 % yeast extract) at 22 °C for five days. The mycelium was fixed in 10 % buffered formalin for 24 h, washed three times in phosphate-buffered saline (PBS) for 10 min, cut into pieces between 1 and 5 mm in size and subjected to three 5-min cycles of sonication using a Branson Sonifier® 450 (Branson Ultrasonics, Danbury). The sample was centrifuged at 500 ***g*** for 5 min and the sediment obtained was washed three times in PBS and centrifuged again at 500 ***g*** for 1 min, which left only small hyphal fragments in the

ACCEPTED MANUSCRIPT

ARTICLE IN PRESS

supernatant. The optical density of the supernatant was then measured in a spectrophotometer at 600 nm and adjusted to a value of 0.1 (equivalent to a concentration of 5 × 10^5 hyphal fragments l^{-1}).

For the zoospore–cyst antigen, a mixture of secondary zoospores and cysts of the pathogenic isolate TRU 8, obtained as described by Fregeneda-Grandes *et al.* (2001), were fixed for 30 min in 4 % glutaraldehyde in 100 mM PIPES buffer [piperazine-1,4-bis-(2-ethanesulphonic) acid] at pH 7. After centrifuging at 500 ***g*** for 10 min, the sediment was washed three times with PIPES and suspended in PBS to give a concentration of around 3 × 10^5 spores ml^{-1}.

Immunization and production of Mabs

Two-month-old female BALB/c mice were injected intraperitoneally with 0.5 ml antigen from the pathogenic isolate TRU 8 mixed 1:1 with Freund's complete adjuvant. They received two boosters every two weeks of 0.5 ml antigen mixed 1:1 with Freund's incomplete adjuvant. In weeks three and five, blood samples were collected from their orbital sinuses so as to quantify the immune response by means of an enzyme-linked immunoassay (ELISA). The mouse with the highest titre was used, being given a final booster dose of 0.5 ml of antigen, half intraperitoneally and half intravenously. Three days later, the mouse was euthanized, and its spleen cells were harvested and fused with SP2 myeloma cells using polyethylene glycol and DMSO (dimethyl sulphoxide) (Hibri-Max® PEG solution, Sigma-Aldrich, St Louis,) at a ratio of 4:1 or 5:1 (splenocytes to myeloma cells) to make hybridomas. After fusion, the cells were diluted to 5 × 10^5 cells ml^{-1} in HAT (hypoxanthine–aminopterin–thymidine) medium: RPMI 1640 medium with GlutaMAX™I and 25 mM HEPES (Gibco™, Invitrogen, Pailey) with 20 % of foetal bovine serum (Sigma-Aldrich), 2 % HAT supplement (Invitrogen), 1 % OPI (oxaloacetate–pyruvate–bovine insulin) media supplement (Sigma-Aldrich) and 100 U penicillin ml^{-1}, 100 µg ml^{-1} streptomycin sulphate, 2.5 µg ml^{-1} amphotericin B. HAT medium was replaced by HT medium, prepared as above but using HT supplement

ACCEPTED MANUSCRIPT

(Invitrogen) instead of HAT supplement after the hybridoma selection was complete, usually 7–10 d after fusion. Quantities of 150 or 200 μl fused, diluted cells were distributed into 96-well tissue culture plates and checked for the production of hybridomas. These were tested using an ELISA 14 d after the fusion, to search for antibodies against *Saprolegnia*. Positive wells were expanded into 24-well tissue culture plates, with a further screening 14 d later using ELISAs and immunofluorescence (IF) assays. Hybridomas that continued to produce specific antibodies were chosen and cloned twice by limiting dilution to obtain monoclonal cell lines. The stable clones were expanded for freezing down and the production of a high-titre supernatant.

A total of five fusions were carried out, in which 26 clones were obtained, but some of them ceased to produce antibodies during clonal expansion or were subsequently lost after they were cryopreserved for later use. Hence five Mabs (named 4H2, 5A2, 18A6, 20B4 and 25B2) were used in the present study to determine their reactivity against the different fungal isolates by IF.

IF assay

A quantity of 20 μl of the appropriate hyphal fragment antigen, prepared as described above, was added to each well of the IF slides (bioMérieux, Marcy l'Étoile), allowed to dry in a laminar-flow cabinet, and fixed with acetone for 10 min. Thereafter, 15 μl of the most suitable dilution of Mab in PBS (1:5 for Mab 4H2, 1:30 for Mab 5A2, 1:20 for Mab 18A6, 1:3 for Mab 20B4, and 1:150 for Mab 25B2) were placed in each well. The slides were incubated in a wet chamber for 1 h at 37 °C and then washed three times with PBS (10 min per wash). A dose of 15 μl anti-mouse conjugated with FICT (Sigma-Aldrich) diluted 1:80 in PBS was added to each well. The slides were incubated for 30 min at 37 °C, washed again, air-dried, and finally observed under a Nikon Eclipse E 400 fluorescence microscope. Wells with hyphal antigens of isolate TRU 8 and 1:100 dilution of mouse polyclonal antiserum and a mouse pre-immune serum were used as

ACCEPTED MANUSCRIPT

positive and negative controls, respectively. Polyclonal antiserum was obtained from BALB/c male mice intraperitoneally inoculated with 0.2 ml of hyphal antigen from the isolate TRU 8 three times, at intervals of three weeks. Wells were considered positive if the hyphal fragments emitted a bright, apple-green fluorescence.

Results

Production of Mabs

Five fusions were performed with spleen cells from mice immunized with hyphal fragment antigen from isolate TRU 8 and fused cells were distributed over 49 culture plates (96-well plates), eight plates in the first fusion, 19 plates in the second, seven in the third, five in the fourth, and ten in the fifth. A total of 1836 hybridomas were obtained. Of these, 225 (12.3 %) were positive in the first screening (17 out of 224 in the first fusion, six out of 360 in the second, 116 out of 672 in the third, 64 out of 480 in the fourth, and 22 out of 100 in the fifth). Of these 225 hybridomas, 58 (25.8 %) were positive in the second screening (13 in the first fusion, six in the second, 24 in the third, nine in the fourth, and six in the fifth), and finally 26 stable clones were obtained after being cloned twice by limiting dilution (nine in the first fusion, three in the second, seven in the third, three in the fourth, and four in the fifth). Some of these 26 clones ceased to produce antibodies or were lost after they were cryopreserved. Hence, in the end, five Mabs (termed 4H2, 5A2, 18A6, 20B4, and 25B2) were obtained. A further two fusions were undertaken, using spleen cells from mice immunized with a mixed suspension of zoospores and cysts or the soluble phase of hyphal antigen from TRU 8, but after the process was completed no positive clones were obtained.

Immunofluoresce analysis

The ability of the five Mabs used in this study to distinguish between the different fungal isolates is shown in Table 2. Four of the Mabs used (4H2, 5A2, 20B4 and 25B2) gave variable results and recognized some of the long-haired *S. parasitica* isolates but also cross-reacted with other *Saprolegnia* species without bundles of hairs and with *Achlya*

ACCEPTED MANUSCRIPT

ARTICLE IN PRESS

sp.; in particular Mab 25B2 recognized all the isolates used with this Mab, except isolate 1AC1, which corresponds to *S. terrestris*.

The other Mab (18A6) did not recognize the *Saprolegnia* isolates without bundles of hairs (except the two *S. hypogyna* isolates) or the *Achlya* sp. isolate. Among the long-haired *S. parasitica* isolates, this Mab was able to differentiate between sexual and asexual isolates, because it recognized 17 out of the 18 asexual isolates (with the exception of isolate 48A, which was only recognized by Mab 25B2) and only three of the 17 sexual isolates. With respect to the morphotype of the isolates, Mab 18A6 recognized all morphotype II isolates, with the exception once again of 48A, but it also reacted with six morphotype I isolates and this reaction was strong in the case of three isolates (Table 2; Fig. 2).

Discussion

Saprolegnia isolates causing lesions to salmonid fish frequently do not develop sexual structures in laboratory cultures. Therefore, they cannot be identified by classical criteria for taxonomy. However, these isolates have certain characteristics, especially the presence of bundles of long hooked hairs in the secondary cyst, indirect germination, and ITS sequences, which enable them to be distinguished from other *Saprolegnia* species. Hence, they are considered to form a coherent separate taxon named *S. parasitica* (Beakes *et al.* 1994, Diéguez-Uribeondo *et al.* 2007). However, there is still some discussion about the correct use of this name (used for the first time by Coker in 1923) because it does not adhere to classical taxonomy (Hughes 1994, Johnson *et al.* 2002: *Biology and Systematics of the Saprolegniaceae*, available at: http://www.ilumina-dlib.org and http://www.uncw.edu/people/padgett). In the present study the ability of five Mabs, raised against a pathogenic *S. parasitica* isolate from brown trout, to distinguish between long-haired *S. parasitica* isolates and other *Saprolegnia* species was evaluated. Four of the Mabs used recognized some of the long-haired *Saprolegnia* isolates but also cross-reacted with the other *Saprolegnia*

ACCEPTED MANUSCRIPT

species without bundles of long hairs, and in addition, two of the Mabs also reacted with *Achlya* sp. The other Mab (18A6) did not recognize *Achlya* sp. or the *Saprolegnia* species without bundles of hairs, but it recognized *S. hypogyna*, which has individual long hairs but no bundles. This fact, and also the results from another study undertaken by the authors showing that *S. hypogyna* has a high percentage of indirect germination and similar ITS sequences to isolates from the *S. parasitica* clade (Diéguez-Uribeondo *et al.* 2007), would seem to indicate that *S. parasitica* and *S. hypogyna* are close relatives. However, this Mab was not able to react with all isolates having bundles of long hairs because it recognized mainly the asexual and not the sexual *S. parasitica* isolates, with a few exceptions. In a similar study, Bullis *et al.* (1996) proved the usefulness of two Mabs for distinguishing *S. parasitica* from *S. diclina* and other *Oomycetes* and unrelated fungi. In general, *S. parasitica* isolates had higher titres than *S. diclina* isolates, but this was not always the case as some *S. diclina* and *S. parasitica* isolates showed similarly low titres. The two Mabs in question also cross-reacted with *S. australis* and *Aphanomyces* sp. but with lower titres.

Cross-reactivity is a frequently reported problem with antibodies raised against *Oomycetes*, which have shown different levels of specificity ranging from isolate to order-specific reactions (Hardham *et al.* 1986, Estrada-García *et al.* 1989, Gabor *et al.* 1993, Bullis *et al.* 1996). Several of these studies have shown that Mabs developed against the zoospore or cyst stage are more specific than those raised against the vegetative hypha. In the present study some problems were encountered in obtaining enough zoospore and cyst antigen for immunization protocols and for use in the immunological assays (ELISA and IFA) to test the hybridomas. Nevertheless, the attempt was made in one fusion to obtain Mabs against a mixed zoopore and cyst suspension. The fused cells were distributed over ten plates, but only seven hydridomas were obtained and they were all negative for specific antibodies in the first screening. Beakes and co-workers (Beakes *et al.* 1994, Beakes *et al.* 1995, Burr & Beakes 1994) obtained five Mabs (from two separate fusions) raised against a mixed

ACCEPTED MANUSCRIPT

ARTICLE IN PRESS

zoospore–cyst suspension of *S. parasitica*. These antibodies were tested against zoospores and cysts but showed cross-reactions to a range of *Saprolegnia* species and could not distinguish *S. parasitica* from *S. diclina*. Furthermore, these antibodies did not react with germ tubes.

In previous studies, we described two different morphotypes in Spanish long-haired *Saprolegnia* isolates, finding a significant difference between the mortality produced by the two morphotypes in natural and experimental infections (Fregeneda-Grandes *et al.* 2000, 2001). The more pathogenic isolates belonged to morphotype II, which had bundles in the secondary cyst with a smaller number and shorter length of hairs and were usually isolated from salmonids with saprolegniosis. Although Mab 18A6 recognized all morphotype II isolates (except one), the distinction between the two morphotypes was not clear because the Mab also reacted with six of the isolates that belonged to morphotype I. With three of them, the reactions were even stronger than those noted with the morphotype II isolates (see Table 2).

It may be concluded that long-haired *S. parasitica* isolates are closely related to other members of the genus *Saprolegnia* and share several antigens, which concurs with Bullis *et al.* (1996). Mab 18A6 seems to recognize an epitope that is expressed mainly in the asexual isolates, which are the isolates most frequently obtained from cutaneous lesions of trout with saprolegniosis. Hence, it may be possible that this antigen is related to the greater pathogenicity of these isolates.

Acknowledgements

This research was supported by grant PB98-0039 from the Spanish Ministry of Education and Culture *(Ministerio de Educación y Cultura)*. The authors thank M.D. Moragues for her valuable help with the monoclonal antibodies techniques and G. Fernández-Bayón for technical assistance with cell cultures. We are grateful to the Local Environmental Service for Leon of the Regional Government of Castile and Leon [*Servicio Territorial de Medio Ambiente de León (Junta de Castilla y León)*] for

ACCEPTED MANUSCRIPT

collaboration in catching the wild brown trout, alevins, and eggs from which the *Saprolegnia* isolates used in this work were obtained.

References

Aller Gancedo JM, Fernández Díez M, 1987. Saprolegniosis en la trucha común (*Salmo trutta*) de los ríos de León (España). *Medicina Veterinaria* **4:** 181-184.

Aller Gancedo JM, Fregeneda Grandes JM, Fernández Díez M, 1999. A method for maintaining stock cultures of *Saprolegnia*. *Abstracts of the EAFP Ninth International Conference on Diseases of Fish and Shellfish*. Rhodes, Greece, pp. 225.

Beakes GW, Wood SE, Burr AW, 1994. Features which characterize *Saprolegnia* isolates from salmon fish lesions—a review. In: Mueller J (ed), *Salmon Saprolegniasis*. US Department of Energy, Bonneville Power Administration, Portland, pp. 33-66.

Beakes GW, Burr AW, Wood SE, Hardham AR, 1995. The application of spore surface features in defining taxonomic versus ecological groupings in oomycete fungi. *Canadian Journal of Botany* **73** (Suppl. 1): S701-S711.

Bruno DW, Wood BP, 1999. *Saprolegnia* and other *Oomycetes*. In: Woo PTK, Bruno DW (eds), *Fish Diseases and Disorders.* Vol. 3*. Viral, bacterial and Fungal Infections*. CABI Publishing, Wallingford, pp. 599-659.

Bullis RA, Noga EJ, Levy MG, 1990. Immunological relationship of the fish-pathogenic oomycete *Saprolegnia parasitica* to other oomycetes and unrelated fungi. *Journal of Aquatic Animal Health* **2:** 223-227.

Bullis RA, Noga EJ, Levy MG, 1996. Production and preliminary characterization of monoclonal antibodies to *Saprolegnia parasitica*. *Mycological Research* **100:** 489-494.

ACCEPTED MANUSCRIPT

ARTICLE IN PRESS

Burr AW, Beakes GW, 1994. Characterization of zoospore and cyst surface structure in saprophytic and fish pathogenic *Saprolegnia* species (oomycete fungal protists). *Protoplasma* **181:** 142-163.

Coker WC, 1923. *The Saprolegniaceae, with Notes on other Water Molds*. The University of North Carolina Press, Chapel Hill. Reprint by Cramer J (1979), Bibliotheca Mycologica, band 20, Vaduz.

Diéguez-Uribeondo J, Fregeneda-Grandes JM, Cerenius L, Pérez-Iniesta E, Aller-Gancedo JM, Tellería MT, Söderhäll K, Martín MP, 2007. Re-evaluation of the enigmatic species complex *Saprolegnia diclina–S. parasitica* based on morphological, physiological and molecular data. *Fungal Genetic and Biology* (in press)

Estrada-Garcia MT, Green JR, Booth JM, White JG, Callow JA, 1989. Monoclonal antibodies to cell surface components of zoospores and cysts of the fungus *Pythium aphanidermatum* reveal species-specific antigens. *Experimental Mycology* **13:** 348-355.

Fregeneda-Grandes JM, Fernández-Díez M, Aller-Gancedo JM, 2000. Ultrastructural analysis of *Saprolegnia* secondary zoospore cyst ornamentation from infected wild brown trout, *Salmo trutta* L., and river water indicates two distinct morphotypes amongst long-spined isolates. *Journal of Fish Diseases* **23:** 147-160 and Erratum *Journal of Fish Diseases* **26:** 313.

Fregeneda-Grandes JM, Fernández-Díez M, Aller-Gancedo JM, 2001. Experimental pathogenicity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), of two distinct morphotypes of long-spined *Saprolegnia* isolates obtained from wild brown trout, *Salmo trutta* L., and river water. *Journal of Fish Diseases* **24:** 351-359.

ACCEPTED MANUSCRIPT

Gabor BK, O' Gara ET, Philip BA, Horan DP, Hardham AR, 1993. Specificities of monoclonal antibodies to *Phytophthora cinnamomi* in two rapid diagnostic assays. *Plant Disease* **77:** 1189-1197.

Hardham AR, Suzaki E, Perkin JL, 1986. Monoclonal antibodies to isolate-, species-, and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*. *Canadian Journal of Botany* **64:** 311-321.

Hughes GC, 1994. Saprolegniasis, then and now: a retrospective. In: Mueller J (ed), *Salmon Saprolegniosis*. US Department of Energy, Bonneville Power Administration, Portland, pp. 3-32.

Miles DJC, Thompson KD, Lilley JH, Adams A, 2003. Immunofluorescence of the epizootic ulcerative syndrome pathogen, *Aphanomyces invadans*, using a monoclonal antibody. *Diseases of Aquatic Organisms* **55:** 77-84.

Noga EJ, 1993. Water mold infections of freshwater fish: recent advances. *Annual Review of Fish Diseases* **3:** 291-304.

Pickering AD, Willoughby LG, 1982. *Saprolegnia* infections of salmonid fish. In: Roberts RJ (ed), *Microbial Diseases of Fish*. Academic Press, London, pp. 271-297.

Seymour RL, 1970. The genus *Saprolegnia*. *Nova Hedwigia* **19:** 1-124.

ACCEPTED MANUSCRIPT

Fig. 1. Secondary cysts with bundles of long hairs (arrows) from *Saprolegnia parasitica*, and without bundles from *S. diclina*. (A) TEM of *S. parasitica* group I isolate 5B8a; (B) TEM of *S. parasitica* group II isolate 70A; (C) phase contrast microscopy image of *S. parasitica* isolate 5B8a; (D) phase contrast microscopy image of *S. diclina* PX16A. Bars = (A–B) 1 μm; (C–D) 5 μm

Fig. 2. Immunofluorescence micrographs showing the reaction of *S. parasitica* Mab 18A6 to hyphal fragment antigen of various *Saprolegnia* isolates. (A) Strong fluorescence with asexual *S. parasitica* group II isolate 2620 A; (B) moderate fluorescence with asexual *S. parasitica* group II isolate TRU 8; (C) no reaction with sexual *S. parasitica* group I isolate 5B8a; (D) strong fluorescence with *S. hypogyna* isolate 1GAP1. Bars = 20 μm

ACCEPTED MANUSCRIPT

ARTICLE IN PRESS

Table 1. Characteristics and origin of the 48 *Saprolegniaceae* isolates

Isolate code	Host or habitat	Bundles of long hairs (cyst morphotype group)[a]	Identification
TRU 3	*Salmo trutta*[b]	Yes (group II)	*Saprolegnia parasitica* [c]
TRU 4	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
TRU 6	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
TRU 8	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
TRU 12	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
48 A	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
70 A	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
146 A	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
154 A	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
200 A	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
2375 A	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
2620 A	*S. trutta*[b]	Yes (group II)	*S. parasitica* [c]
2M5e	River water	Yes (group I)	*S. parasitica* [c]
3B15b	River water	Yes (group I)	*S. parasitica* [c]
5M16a	River water	Yes (group II)	*S. parasitica* [c]
7B4a	River water	Yes (group II)	*S. parasitica* [c]
7M13a	River water	Yes (group II)	*S. parasitica* [c]
9B5f	River water	Yes (group I)	*S. parasitica* [c]
32 C	*S. trutta*[b]	Yes (?) [d]	*S. parasitica* [e]
172 A	*S. trutta*[b]	Yes (?) [d]	*S. parasitica* [e]
PI484	*S. trutta*[f]	Yes (NT)	*S. parasitica* [e]
2451 A	*S. trutta*[g]	Yes (group I)	*S. parasitica* [e]
2451 B	*S. trutta*[g]	Yes (group I)	*S. parasitica* [e]
2451 C	*S. trutta*[g]	Yes (group I)	*S. parasitica* [e]
1B1c	River water	Yes (group I)	*S. parasitica* [e]
1B10c	River water	Yes (group I)	*S. parasitica* [e]
1M5a	River water	Yes (group I)	*S. parasitica* [e]
2B2a	River water	Yes (group I)	*S. parasitica* [e]
2M17a	River water	Yes (group I)	*S. parasitica* [e]
5B8a	River water	Yes (group I)	*S. parasitica* [e]
6M3c	River water	Yes (group I)	*S. parasitica* [e]

ACCEPTED MANUSCRIPT

ARTICLE IN PRESS

7M17b	River water	Yes (group I)	*S. parasitica* [e]
9M8c	River water	Yes (group I)	*S. parasitica* [e]
9M8d	River water	Yes (group I)	*S. parasitica* [e]
9M9c	River water	Yes (group I)	*S. parasitica* [e]
PY1B	Egg of *S. trutta* [h]	No	*S. diclina*
PY2	Alevin of *S. Trutta*[h]	No	*S. diclina*
PY5E1	Egg of *S. trutta* [h]	No	*S. diclina*
PX16A	Egg of *S. trutta* [h]	No	*S. diclina*
PX19-2	Egg of *S. trutta* [h]	No	*S. diclina*
1FP1	River water	No	*S australis*
11AP1	River water	No	*S. australis*
7BP1	River water	No	*S. ferax*
10BP1	River water	No	*S. ferax*
1GAP1	River water	No	*S. hypogyna*
PX25	Egg of *S. trutta* [h]	No	*S. hypogyna*
1AC1	River water	No	*S. terrestres*
4CO3	River water	No	*Achlya* sp.

[a]Cyst morphotype group: see Fregeneda-Grandes *et al.* 2000.
[b]Cutaneous lesions of wild fish with saprolegniosis.
[c]Asexual isolates formerly identified as *Saprolegnia* sp.
[d]Intermediate values between cyst morphotype group I and II.
[e]Sexual isolates (only at 7 °C) formerly identified as *S. diclina* Type 1.
[f]Cutaneous lesions of cultured fish with saprolegniosis.
[g]Mucus of wild brown trout without saprolegniosis; death due to water pollution.
[h]Cultured eggs and alevins colonized by *Saprolegnia* spp.

NT, not tested.

ACCEPTED MANUSCRIPT

Table 2. Ability of the five anti-*Saprolegnia parasitica* monoclonal antibodies (Mabs) used in the present study to distinguish between various *Saprolegnia* spp. and *Achlya* sp. isolates as detected by immunofluorescent assay

Isolates	Bundles of long hairs (cyst morphotype group) [a]	Mab 4H2 (diluted 1:5)	Mab 5A2 (diluted 1:30)	Mab 20B4 (diluted 1:3)	Mab 25B2 (diluted 1:150)	Mab 18A6 (diluted 1:20)
***Saprolegnia parasitica* (asexual)**						
TRU 3	Yes (group II)	+	+/−	+/−	+	+
TRU 4	Yes (group II)	+	+	+	+	+
TRU 6	Yes (group II)	−	+/−	−	+	+
TRU 8	Yes (group II)	+	+/−	+/−	+	+
TRU 12	Yes (group II)	+	+/−	−	+	+
48 A	Yes (group II)	−	−	−	+	−
70 A	Yes (group II)	NT	NT	NT	NT	+
146 A	Yes (group II)	NT	NT	NT	NT	+
154 A	Yes (group II)	NT	NT	NT	NT	+
200 A	Yes (group II)	NT	NT	NT	NT	+
2375 A	Yes (group II)	+	−	+/−	+	+
2620 A	Yes (group II)	NT	NT	NT	NT	++
2M5e	Yes (group I)	+/−	−	−	+/−	++
3B15b	Yes (group I)	+	−	−	+/−	++
5M16a	Yes (group II)	+	+	−	+	+
7B4a	Yes (group II)	+/−	+/−	−	+	+
7M13a	Yes (group II)	+/−	+	+/−	+	+
9B5f	Yes (group I)	+/−	−	−	+/−	++
***S. parasitica* (sexual)**						
32 C	Yes (?) [b]	−	−	−	+/−	−
172 A	Yes (?) [b]	−	−	−	+	−
PI484	Yes (NT)	NT	NT	NT	NT	−
2451 A	Yes (group I)	NT	NT	NT	NT	−
2451 B	Yes (group I)	+/−	−	+/−	+/−	−
2451 C	Yes (group I)	NT	NT	NT	NT	+
1B1c	Yes (group I)	NT	NT	NT	NT	+
1B10c	Yes (group I)	NT	NT	NT	NT	−
1M5a	Yes (group I)	NT	NT	NT	NT	+

ACCEPTED MANUSCRIPT

2B2a	Yes (group I)	NT	NT	NT	NT	−
2M17a	Yes (group I)	+	+	+/−	+	−
5B8a	Yes (group I)	+	+	+	+	−
6M3c	Yes (group I)	+/−	+/−	+/−	+/−	−
7M17b	Yes (group I)	NT	NT	NT	NT	−
9M8c	Yes (group I)	+/−	+/−	−	+/−	−
9M8d	Yes (group I)	NT	NT	NT	NT	−
9M9c	Yes (group I)	NT	NT	NT	NT	−
S. diclina						
PY1B	No	NT	NT	NT	NT	−
PY2	No	NT	NT	NT	NT	−
PY5E1	No	NT	NT	NT	NT	−
PX16A	No	NT	NT	NT	NT	−
PX19-2	No	NT	NT	NT	NT	−
S. australis						
1FP1	No	+/−	+/−	+	+/−	−
11AP1	No	+/−	+	+	+/−	−
S. ferax						
7BP1	No	−	−	+/−	+/−	−
10BP1	No	+	+	+	+/−	−
S. hypogyna						
1GAP1	No	+/−	+/−	−	+/−	++
PX25	No	NT	NT	NT	NT	+
S. terrestris						
1AC1	No	−	+/−	+/−	−	−
Achlya sp.						
4CO3	No	+/−	−	−	+/−	−

[a]Cyst morphotype Group: see Fregeneda-Grandes *et al.* 2000.
[b]Intermediate values between cyst morphotype Group I and II.

Fluorescence: ++ = strong; + = moderate; +/− = weak; − = negative.
NT, not tested.

AUTHOR QUERIES

ACCEPTED MANUSCRIPT

ARTICLE IN PRESS

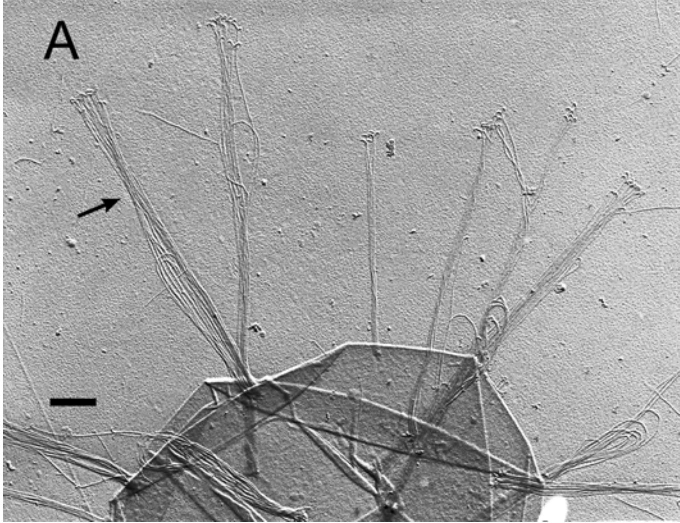
PROVIDE STATE FOR: Branson Ultrasonics, Sigma-Aldrich, Invitrogen

DEFINE: FICT

Diéguez-Uribeondo et al 2007: UPDATE IF POSSIBLE

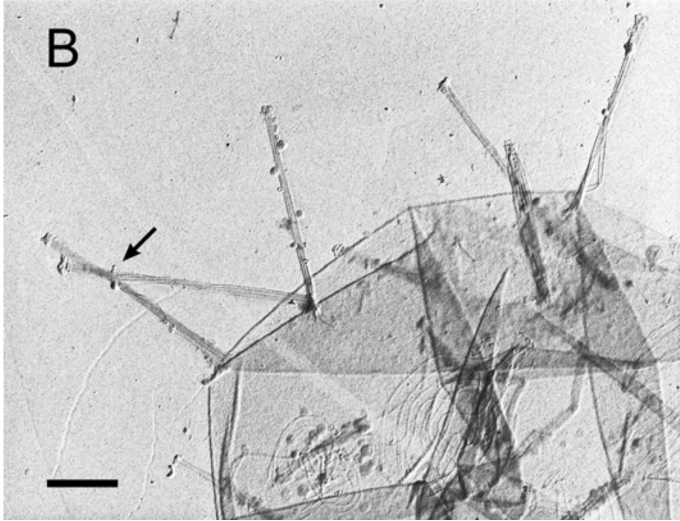
PROVIDE FIRSTNAMES OF PEOPLE ACKNOWLEDGED

ACCEPTED MANUSCRIPT

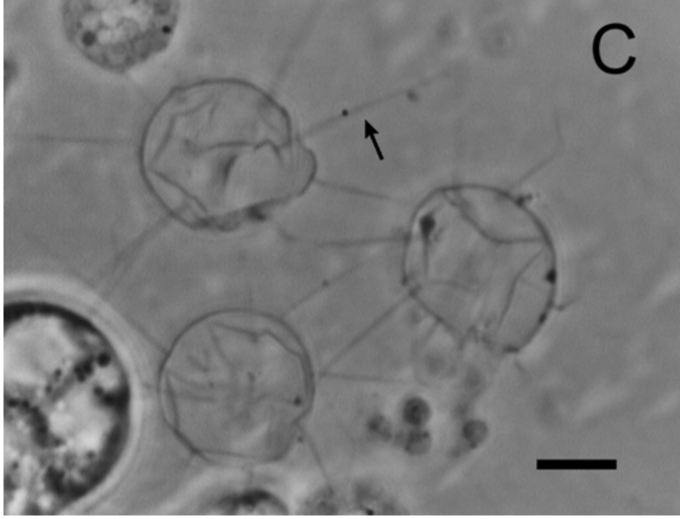


ACCEPTED MANUSCRIPT

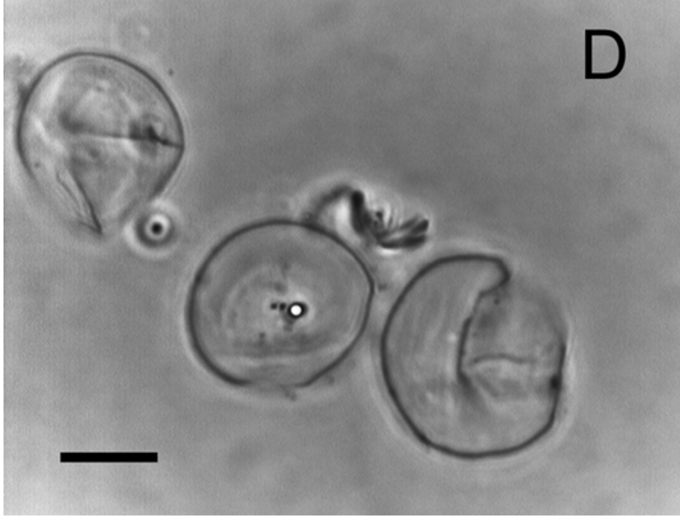
ARTICLE IN PRESS



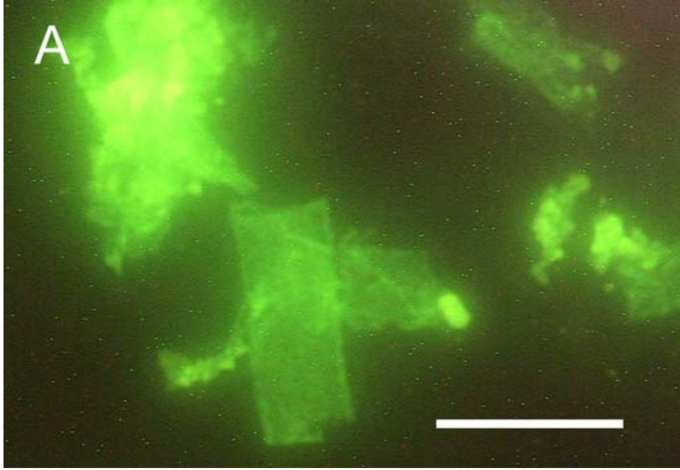
ACCEPTED MANUSCRIPT



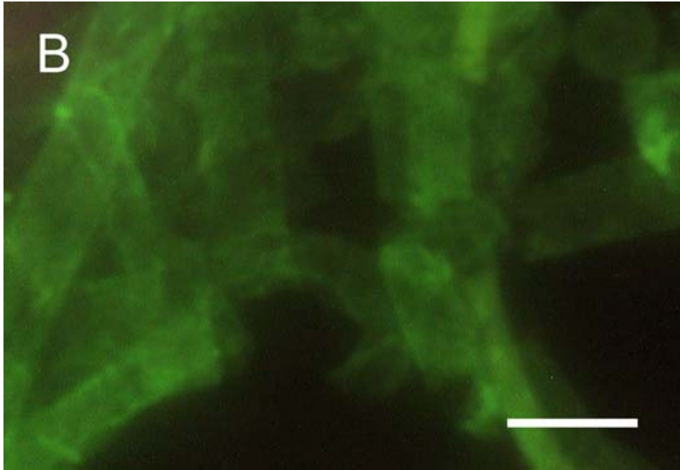
ACCEPTED MANUSCRIPT



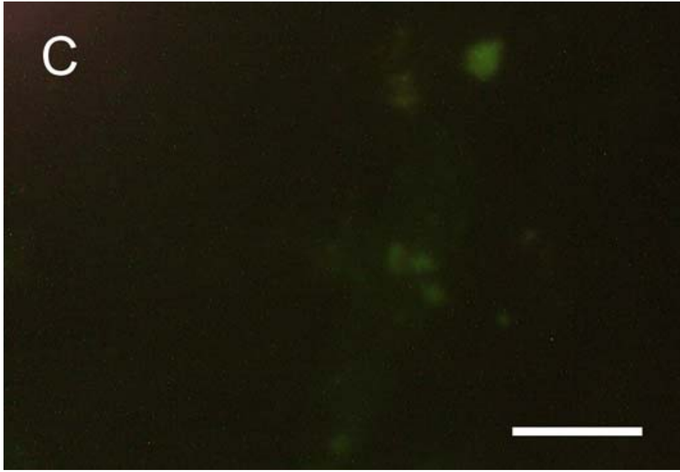
ACCEPTED MANUSCRIPT



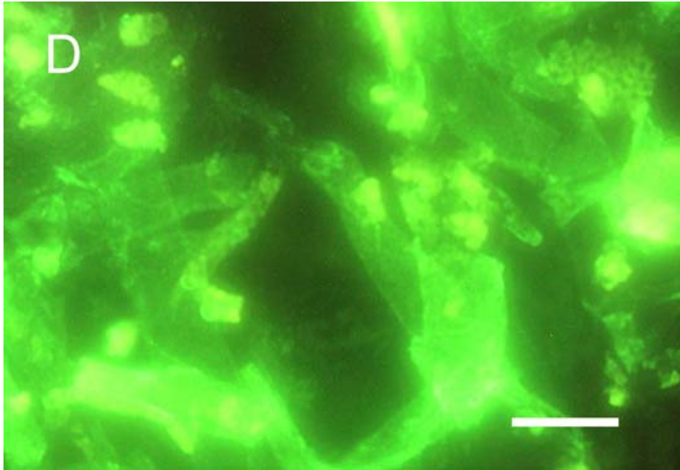
ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT



ACCEPTED MANUSCRIPT