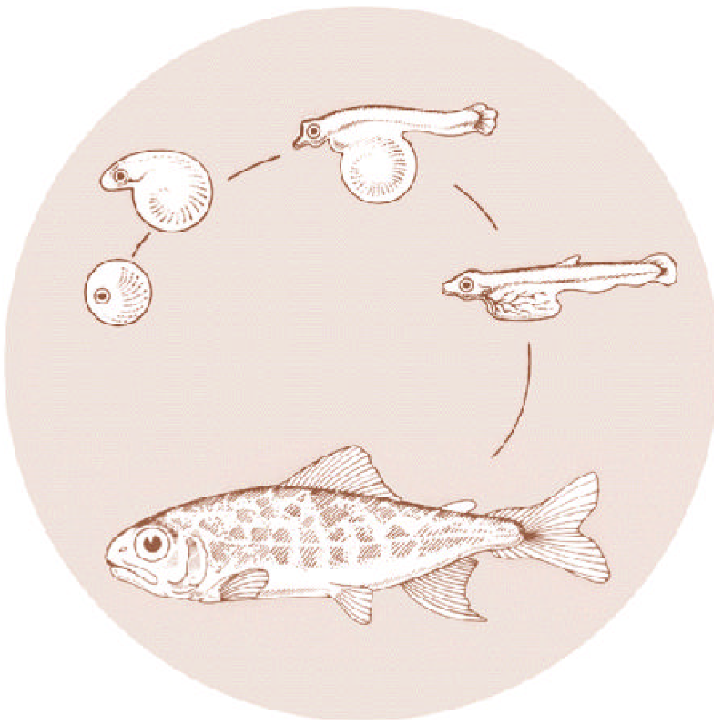
SAPROLEGNIA SPP.PATHOGENIC IN CHINOOK SALMON, PERIOD OF REPORT JANUARY 15, 1993 TO JUNE 14, 1996

Final Report



DOE/BP-02836-2

This report was funded by the Bonneville Power Administration (BPA), U.S. Department of Energy, as part of BPA's program to protect, mitigate, and enhance fish and wildlife affected by the development and operation of hydroelectric facilities on the Columbia River and its tributaries. The views of this report are the author's and do not necessarily represent the views of BPA.

This document should be cited as follows:

Whisler, Howard C. - Dept. of Botnay - University of Washington, Identification of Saprolegnia SPP.Pathogenic in Chinook Salmon, Final Report, Period of Report: January 15, 1993 to June 14, 1996, Report to Bonneville Power Administration, Contract No. 1990BP02836, Project No. 199006100, 58 electronic pages (BPA Report DOE/BP-02836-2)

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IDENTIFICATION OS SAPROLEGNIA SSP. PATHOGENIC IN CHINOOK SALMON

Final Report
Period of Report:
January 15, 1992 to June 14, 1996

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Project No. 90-61
Contract No. DE-AC79-90BP02836

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ACKNOWLEDGMENTS

This project depended on the direct support and assistance of many experts. Dr. George J. Mueller was central to the planning and execution of all aspects of the project. Other important members of our laboratory included Hong Tian, Peter Werner, and Kenneth Mc Nabb. We appreciate the help and collaboration with Drs. Rusty Rodriquez and Gayle Brown and the use of the facilities of the N.W. Biological Science Center of the National Biological Survey.

We are indebted to many members of the hatchery and fish-health community for their patience and assistance. We must especially thank Kevin Amos, Paul Peterson, Mark DeCew, Tami Black, Pat Chapman, and Dan Barrett.

Our efforts in DNA fmgerprinting were made possible by the generosity and time of Professor John Taylor and his colleagues including; Drs. Eric Swann, Austin Burt, Mary Berbee, Dave Carmean, Greg Saenz, Dee Carter and Tom Bruns.

We must also acknowledge that our work built on earlier studies by Dr. Gil Hughes, and his students, the members of the "Windermere School" of saprolegniasis, and the other contributors to the seminar on "*Saprolegnia in* Salmon".

The project was made feasible by the Bonneville Power Administration and we appreciated the constant support and council of Rick Westerhof and Alan Ruger.

ABSTRACT

This project has developed procedures to assess the role of the fungal parasite, ***Saprolegnia*** in the biology of salmon, particularly adult Chinook, in the Columbia River Basin. Both morphological and DNA "fingerprinting" surveys reveal that ***Saprolegnia parasitica*** (= *S.* ***diclina,*** Type I) is the most common pathogen of these fish. In the first phase of this study 92% of 620 isolates, from salmon lesions, conformed to this taxa of ***Saprolegnia.*** In the current phase, we have developed variants of DNA fingerprinting (RAPD and SWAPP analysis) that permit examination of the sub-structure of the parasite population. These results confirm the predominance of ***S. parasitica,*** and suggest that at least three different sub-groups of this fungus occur in the Pacific N.W., USA. The use of single and paired primers with PCR amplification permits identification of pathogenic types, and distinction from other species of the genus considered to be more saprophytic in character. A year's survey of saprolegniaceous fungi from Lake Washington indicated that the fish-pathogen was not common in the water column. Where and how fish encounter this parasite can be approached with the molecular "tags" identified in this project.

Fish stressed either by net handling or by social interaction are susceptible to our standard isolate, 292-l. In contrast to other studies, sexual reproduction was not rare in our fish-isolates. Hemp seed cultures in the laboratory tended to yield abortive oospores, but if the pathogenic isolate was permitted to develop on its killed, salmon host in a constant water stream, apparently normal oogonia and oospores were abundantly produced within two weeks.

The hyperparasite ***Woronia*** *polycystis* was isolated into monoxenic culture from *S. parasitica* from three different hatcheries. Cystosori are produced in these cultures and the potential for biological control of ***Saprolegnia*** with this obligate parasite is available for study.

INTRODUCTION

I. BACKGROUND

The water mold, ***Saprolegnia*** (Fig. 1) is well known as a fungal parasite of fish. It may be responsible for rapid destruction of eggs or extensive external necrosis on adult salmon. These lesions, often carrying a free mass of fungal mycelium, have been recorded for over 250 years. A brief review of saprolegniasis in fish, however, reveals a startling lack of basic information on these obvious molds, and the disease they incite in their host animals. This gap in knowledge relates to several factors that bear on this research project.

There has been a prolonged debate, dating from the great 1880 salmon plague in England, as to whether *Saprolegnia* acts as a primary or secondary disease agent (Huxley, 1882; Rucker, 1944; Neish and Hughes, 1980; Hatai, 1994). This argument gradually received less and less energy following the discovery and widespread use of the effective chemical control agent, malachite green. The situation is now changing. Malachite green is recognized as a toxic compound and is essentially banned in the United States. Formalin, the alternative choice, has its own problems (e.g. potential toxicity to handlers; cost of container disposal).

On the fungal side, there are relatively few studies of ***Saprolegnia*** spp. in fish. Tiffney (1939); O'Bier (1960); Neish (1977); Willoughby (1978); and Hatai (1994) all point to the existence of special strains of the fungus that parasitize different types of fish. These strains are distinct from the saprophytic water molds that may be isolated from almost all ponds, streams and lakes.

The parasitic strains can be repeatedly isolated from sahnonid fish, and both Tiffney (1939) and Neish (1976) have produced experimental data indicating that some strains of ***Saprolegnia*** may attack uninjured salmon. These mycological studies also point out the difficulties faced in studying ***Saprolegnia*** in fish. This water mold is a member of the ***Oomycetes***, a distinct group of fungi that is, now commonly, associated with the Protista and not the true fungi (Margulis et al., 1990). Although these organisms, with their biflagellate spores and unusual cell walls, are obviously different from other molds, mycologists have struggled to fmd adequate criteria to classify the different taxa within the class, Oomycetes, itself. Taxonomic differentiation, from the ordinal to the sub-specific level, is difficult and under constant revision (Dick, 1990).

In the case of the Saprolegniaceae, the family that includes most fish pathogens, the generic distinctions are mainly based on the nature of asexual reproduction, which we now know may be influenced by a variety of environmental factors such as temperature, O_2, CO,, and light (Salvin, 1941; Scott, 1956). Identification at the species level, in contrast, relies on the morphology of the sexual phase. Regrettably, watermolds isolated from salmon and other aquatic animals typically lack a sexual phase. Coker (1923), an early student of these fungi, pragmatically decided to create a special taxon, *Saprolegnia parasitica* for these asexual, unidentifiable water molds. Subsequent workers found occasional sex organs in their isolates, especially when the latter were cultured under special conditions, and the species, S. *parasitica*, was redefined (Kanouse, 1932). Contemporary reviews of this problem (Hughes, 1994; Neish and Hughes, 1980; Seymour, 1970) have forcibly pointed out that we are still suffering from a dualistic view of the causal agent of saprolegniasis in fish. Neish (1976) and Willoughby (1978) responded to this problem by studying multiple characters of numerous isolates from parasitized salmon. Their results established the existence of different biotypes, showing differing patterns of specificity and pathogenicity toward

the host fish. Most isolates were asexual, but sexual reproduction could often be induced by special treatment. Neish (1976) concluded that the taxon, ***SaproZegnia parasitica*** should be eliminated and the main salmon parasites be included in a broadly expanded ***S. diclina* Humphrey.** The problem now becomes: How do we recognize the sub-species of ***S. diclina*** that are specific pathogens of salmonid fish? With the morphological traits being so variable and sexual reproduction so limited, the identification of any fungus isolated from spring and summer chinook salmon would be of questionable value.

It is, therefore, essential that new tools be employed in the study of saprolegniasis. Willoughby (1978) has suggested that the fine structure of zoospore cyst would be useful in this problem. Peduzzi and Bizzozero (1977) have used serological tests with four species of ***Saprolegnia.*** Neish and Green (1976) introduced the use of nuclear and satellite DNA base composition to the taxonomy of ***SaproZegnia.*** Beakes and colleagues (Beakes and Ford, 1983; Wood, 1988; Beakes, Wood and Burr, 1994) employed isozyme analysis to study ***S. parasitica*** populations in Great Britain. Their important studies support the hypothesis that a type of ***SaproZegnia*** with long hooks on its secondary cysts, is a, if not the most, common ***Saprolegnia*** on salmon. The recent advances in DNA "fingerprinting" techniques argue for its use as the tool of choice for future identification of ***Saprolegnia*** isolates.

In summary of the current situation, the preferred chemotherapeutant has been banned from routine use. More intensive fish handling and aquaculture will probably enhance parasitism by ***Saprolegnia*** species. Knowledge and identification of fungal isolates that attack spring and summer chinook salmon are needed for the proper assessment of new control approaches. Ready knowledge of the presence or absence of salmon-specific strains of ***Saprolegnia*** could aid hatchery personnel evaluate growing conditions or measure effectiveness of control measures.

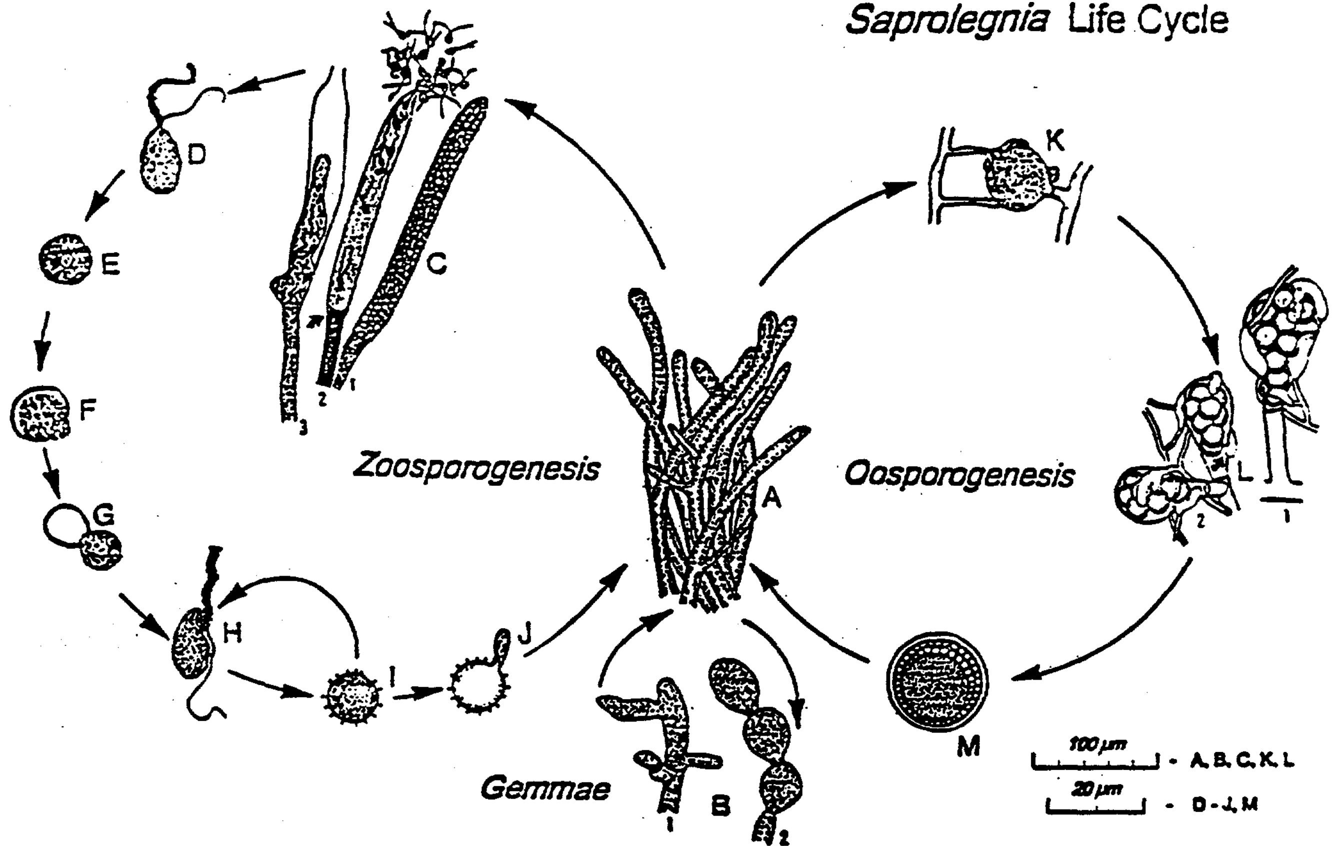
II. PRESENT PROJECT

Our investigation into saprolegniasis may be divided into three phases:

Phase One: Isolation and culture of strains of ***Saprolegnia*** parasitizing salmon.

Phase Two: Organization of a special seminar on "***Saprolegnia*** in Salmon", August 8, 1992 at the Annual Meeting of the Mycological Society of America in Portland, OR. This was followed by preparation of the proceedings and compilation of a comprehensive bibliography on saprolegniasis (with Drs. Hughes and Choi).

Phase Three: Identification of strains of ***Saprolegnia*** parasitizing salmon in the Columbia River Basin. This required the development of new diagnostic tools to recognize different strains of ***S. parasitica.*** Preliminary studies on pathogenesis and bio-control were also initiated.



HUGHES

FIGURE 1. Typicai *Saprolegnia* Life Cycle; **A)** Vegetative **mycelium (2n),** B) **Gemmae; B-1, irregularly shaped gemmae, B-2, catenulate chain of gemmae; C) Zoosporangia. C-1,** nearly **mature, zoospores visable internally;** C-2, Mature **zoosporangium,** releasing **primary zoospores;** c-3, **Empty zoosporangium with** new **zoosporangium** forming by **internal proliferation;** D) Primary, **pyriform zoospore; E)** Encysted **primary zoospore; F,G) Germinating** primary **cyst; H) Secondary, reniform zoospore,** note insertion of **flagella; I) encysted secondary zoospore,** note hooked projections on **surface** of **cyst; J) Germinating secondary cyst.** Note: **Secondary zoospores may** go through **several cycles** of **encystment to produce the hyphal element seen here; K) Immature oogonium** with **immature diclinous antheridia; L), Mature oogonia,** with **mature oospores (zygotes).** Note **fertilization** tube at arrow, **L-2; M)** Mature, subcentric oospore.

Phase One: January 1991 - June 1992

The primary emphasis of the first phase of this project was the isolation, culture, and characterization of fungi in sahnonid fish, particularly ***Saprolegnia*** in adult spring chinook. Over 1000 isolates of ***Saprolegnia*** and other fungi were taken from adults, juveniles and eggs from hatcheries throughout the Columbia River system. These fungi were isolated into an axenic culture and preserved in liquid nitrogen for future use and reference. Approximately 300 of the isolates were selected for intensive characterization in terms of morphological and physiological variation. The current systematics of ***the*** *Saprolegniales* is based on the morphology of the sexual phase of these water molds. Unfortunately, the fish pathogenic isolates, as mentioned above, are notorious for their recalcitrant sex in pure culture. Their identification, therefore, must rely on other morphological features, which are of limited reliability.

This kind of morphological information, however, is essential as a first step in understanding variation in these salmon pathogens. Comparative physiology of the isolates was detailed by examining relative protease production and growth rates. The results of this primary phase are presented in our last final report to Bonneville Power Administration (BPA) and in our contribution to the "Special Seminar of ***Saprolegnia*** in Salmon" (Mueller and Whisler, 1994).

Phase Two: The Seminar: Saprolegniasis iu Salmon, August 8, 1992

This seminar, supported by Bonneville Power Administration, was held in Portland, OR in 1992. in conjunction with the annual meeting of the Mycological Society of America. We organized this conference to bring together for the first time, international experts on saprolegniasis in salmon. It is a regrettable fact that the United States, in contrast to England, Canada, Japan, Norway, and Denmark, has devoted little energy to research on this subject. The proceedings of the symposium, was edited by Dr. Mueller. The proceedings also include a bibliography of all known papers on saprolegniasis (Hughes, Mueller and Choi, 1994). In addition to a visit to the Bonneville Fish Hatchery the participants had the opportunity to reflect on outstanding problems, and their thoughts are included in the Proceedings as a list of current research needs (Mueller, Chap. 12, 1994).

Phase Three: January 15, 1992 - June 14, 1996 (Present Report Period)

Several points were frequently emphasized during the seminar on Saprolegniasis in Salmon:

- Identification. The variable morphological and physiological characteristics of ***Saprolegnia*** make identification of fish pathogens difficult. New molecular finger-printing techniques are needed.
- Pathogenicity. Evidence is now accumulating that some strains of ***Saprolegnia are*** actively pathogenic to stressed salmon. Fish in the Columbia River Basin are encountering periods of stress throughout their development from eggs to adults. Saprolegniasis is a growing concern and should not be treated, as in the past, as a problem that will go away if we just raise fish in good conditions and protect them from bacteria and viruses.

- Control. Alternatives to the fungicide, malachite green and formalin, are needed. Malachite green is currently prohibited in the U.S. It has been replaced by formaldehyde which is expensive, has a relatively narrow safety range for the fish, and has special handling problems.

  Biological control approaches are being investigated in Denmark and Japan. ***Saprolegnia*** inhibiting bacteria have been isolated from fish-slime and their potential use in fish culture is being investigated.

- Basic research in reproductive biology of *Saprolegnia.* Research into the basic biology of *Saprolegnia* and other fish-pathogenic fungi is needed. In contrast to fungal pathogens of crop plants, little time has been devoted to the mechanisms of zoosporogensis and host-colonization of fish disease fungi. ***Saprolegnia*** is generally considered to be a superficial parasite, but there is now abundant evidence to confirm its invasive capacity in some situations. Histological studies of fungal host-tissue interactions are needed.

The third phase of our project was directed toward the four problems listed above, with emphasis on the development of molecular techniques to identify ***varieties*** of ***S. parasitica*** and other pathogenic strains of *Saprolegnia.*

IDENTIFICATION

I. INTRODUCTION TO MOLECULAR " FINGERPRINTING" OF *SAPROLEGNIA*

The genus ***Saprolegnia*** is represented in fresh water habitats through out the world. Several species, ***Saprolegnia diclina, S.*** *ferax*, ***S. hypogyna,*** and ***S. parasitica,*** are commonly reported from many widely dispersed geographical locations and also from lesions of fishes. ***Saprolegnia parasitica,*** a poorly characterized species, has been reported as a primary pathogen of salmons and is the primary object of this report. ***Saprolegnia parasitica,*** difficult to distinguish from *S.* ***diclina,*** has been reported to have secondary zoospore cysts ornamented with long bundles of hooks. In the first phase of this study we found that the secondary zoospore cysts of 557 out of 605 isolates (92 percent) obtained from both juveniles and adults of various species of salmonids had the long bundles of hooks (Mueller and Whisler, 1993). The availability of a large collection of ***Saprolegnia parasitica*** isolates afforded the opportunity to compare local interspecific variation in this cosmopolitan species. The existence of separate populations of *S.* ***parasitica may*** help to explain the anecdotal evidence that different out breaks of saprolegniasis vary in pathogenicity. We were also able to extend the study to a limited extent and make comparisons with other species reported from fish lesions and to locations outside of the Columbia River Basin.

Although the majority of our isolates from the Columbia River Basin conformed to the taxon ***S. parasitica*** (sensu ***S. diclina*** Type I, Willoughby, 1978; Neish, 1976), it is important to note that some of the fungi isolated from salmon did not display uniform bundles of long hooks on their cysts, and the nature of variation of these pathogens is largely unknown

The development of new approaches for the identification of ***strains*** of ***Saprolegnia*** parasitic in salmon was given high-priority by all participants in the 1993 symposium on "Saprolegniasis in Salmon".

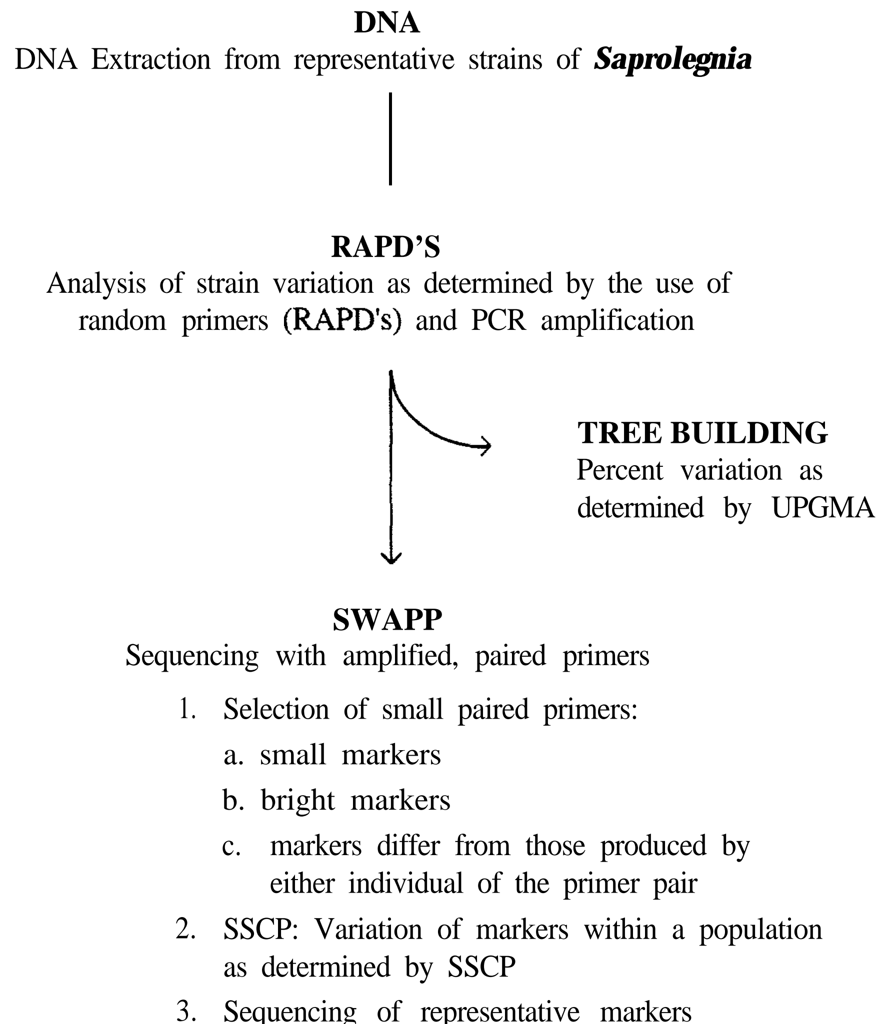
Our response to this need was to develop an approach to DNA "fingerprinting" that would, hopefully, be sensitive enough to:

- detect variation at the sub-specific level
- provide insight as to the degree of variation within the genus
- provide information as to clonal versus recombinational (sexual) development in natural populations
- provide the basis for the design of specific probes for the identification of pathogenic strains of ***Saprolegnia.***

After considering a variety of methods including isozymes, restriction fragment length polymorphisms (RFLP) analysis, and 18S rRNA internal transcribed spacers (ITS) analysis, we elected the approach outlined below:

II. RESEARCH PLAN

A. Research Flow Outline



This relatively complex approach was tied to the current lack of molecular information on ***Saprolegnia*** and the special opportunities presented by a sabbatical leave of the principal investigator (PI) in the laboratory of Dr. John Taylor at the University of California, Berkeley. Dr. Taylor and colleagues were in the process of developing a new approach to‘ the population biology of fungal parasites of ***animals (e. g. Coccidioides*** *immitis* in man). They named their technique "SWAPP" for sequencing with amplified primer pairs (Burt et al., 1996). The advantages and limitations of this strategy will be discussed in the appropriate, following sections.

B. A Brief Description of the Flow Outline Presented Above

1 . RAPD's

For the comparison of populations the use of random amplified polymorphic DNA (RAPD) has recently become available. RAPD analysis appears to be a simple and fast method of measuring genetic variability within populations. It relies on the ability of a relatively small primer to amplify, via PCR, short sections of the genome. These units may then be separated with gel electrophoresis and visualized with ethidium bromide. Reproducibility requires the scoring of only bright markers and careful standardization of technique. This method of analysis has been successfully used on numerous fungal pathogens of insects (Bridge et al., 1993), humans (Bostock et al., 1993), plants (Goodwin and Annis, 1991), and mycorrhizal fungi (Jacobson et al., 1993).

2. SWAPP: A brief summary

Step a. Identification of primer-pairs that produce small (300 base pair) segments of **the genome.**

Markers selected by paired primers migrate in a gel at a rate different from markers amplified with a single primer alone (Fig. 6B). The use of two, paired primers will subsequently permit sequencing of the genome segment.

Step b. Screening against target population.

Paired primers that yield DNA fragments with the right size and brightness are then tested against the population of interest. If the marker is found in all members of interest, but displays some variability toward more distantly related fungi (Fig. 6A), it is a candidate for SSCP screening.

Step c. SSCP (Single-strand conformational polymorphism).

This well established technique depends upon the unique shape that single stranded DNA pieces may take when they are isolated from their complementary strand. When the DNA segment, that has been selected by the paired-primers, is heated, the two, now isolated, single strands will take on a shape unique to their base sequence. If they are then subjected to gel - electrophoresis under conditions that maintain their single strand conformation, the strands will show differing mobility's due to their changed shape. This technique is sufficiently sensitive to reveal single base changes. The SWAPP protocol uses SSCP as a mechanism to screen the population for mutations in the set of common markers selected by the paired-primers in Step 2a. Representative markers are then candidates for base sequence analysis.

Step d. Sequencing

Small DNA segments that have two different ends, as provided by the use of primer-pairs, are candidates for standard base sequencing. If only one primer had been used, as is the case in the standard RAPD process, both ends of the amplified segments would be similar, resulting in mixed synthesis of pieces of both strands.

Once the base sequence is determined for different sets of markers in the population, affiity trees would yield information on relatedness, and whether the population is developing as an asexual clone, or through some kind of recombinational pathway. The latter is of some interest since ***S. parasitica*** is generally assumed to lack normal sexual reproduction.

Sequenced segments of DNA could also be used to identify pathogenic strains of S. ***parasitica*** in both laboratory and field situations.

C. 18S rRNA Sequence

Although this project was focused on sub-specific variation ***in S. parasitica,*** the PI took the opportunity offered by the special facilities and training available in Dr. Taylor's laboratory to initiate sequencing of the 18S rRNA gene from our standard isolate 292- 1. The information gained from such a sequence could aid ***in*** defining the genus ***Saprolegnia,*** and interpreting the relationships of salmonid parasites to other species in the group.

At the present time the best available sequence ***in*** the gene bank is for *Achlya ambisexualis*, a non-pathogenic cousin of ***Saprolegnia.***

III. MATERIALS AND METHODS

A. Fungal Isolates

The origin of the isolates of ***Suprolegnia*** used in these studies are summarized in Table 1. The method of isolation and descriptions of growth rate determination and morphology have been reported elsewhere (Mueller and Whisler, 1994).

B. Isolation of DNA

Fungal cultures were cultured for five to eight days at 20°C in shake cultures containing 75 mls of media designed specifically for ***Suprolegnia parasitica*** (Powell et al., 1971). The cultures were drained on a miracloth filter (Calbiochem), rinsed once with distilled water, the miracloth was folded with the mycelium inside and the remaining water pressed out by hand between absorbent towels. The rinsed mycelium was freeze dried, finely ground in liquid nitrogen, and then the DNA was extracted in accordance with Rodriguez and Yoder (1991) and Rodriguez (1993). The DNA concentration was equilibrated by estimation with known standards on ethidium bromide gels.

C. RAPD's: Amplification and Gel Electrophoresis

Reactions for the first study were carried out in a 25 µl volumes, containing 13.5 µl H_20, 2.5 µl 50% glycerol, 2.5 µl PCR Reaction Buffer, 2.5 µl dNTP's, 0.25 µl TAQ, 2.0 µl Primer (Operon), and 2.0 µl template DNA. The reference template was the DNA extracted from isolates

292-l and 170-3. Isolates 292-l and 170-3 were selected as representatives of two populations studied in the first phase of this project (Mueller and Whisler, 1993). These isolates, 292-l and 170-3, were cultured several times during the study and were used to test reproducibility. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). Details of our protocol are presented as Appendix 1. The 10-mer nucleotide, random primers used in this study were supplied by Operon Technologies Inc. and selected from the D kit. For the second part of the study, primers selected from the C and E kit were added. Amplification reactions were carried out in a 50 µl volume with the reagent proportions used in the first study. Amplification products from both studies, plus a 1KB reference ladder were electrophoretically separated in a 2% agarose gel prepared in TBE buffer. Gels were stained with ethidium bromide. Gels were photographed over a UV trans-illuminator using a Polaroid MP4 camera and Polaroid Type 55 black and white film.

D. Data Analysis

Bright reproducible bands were scored as 1 for presence and 0 for absence. Jaccard's Coefficients were calculated on the resulting matrix. An unweighted pair-group arithmetic average (UPGMA) analysis performed on the matrix resulting from the Jaccard's Coefficients calculations was used to construct the dendrogram. RAPD analysis may be biased if the number of bands is significantly different for any isolate. Anova analysis of the number of bands per isolate indicated that the probability at the 99% level failed to reject the hypothesis that all isolates are equal. The statistical analyses were carried out with Systat 5 for the Macintosh (Systat, Inc.).

E. SWAPP

1. Selection of small, paired-primers

The use of random primers had two objectives. First, they could be used to select markers for direct comparison of populations of *Saprolegnia* via the RAPD approach. Secondly, selected matched pairs of primers could be used to find small genome segments that were common to all members of the population appropriate for base sequencing.

The search for small paired-primers used the same electrophoretic gels described above. Individual primers from a set of 12 different 10-mer primers (Sets: C, D, and E) were run individually and against each other.

Pairs of primers were selected if they provided small, bright markers at a position that differed from either of the individual primers (See Fig. 6B). The initial search utilized genomic DNA from Isolates 292-l and 170-3, our standard isolates. If a pair showed promise, it was then tested against a selected set of *Saprolegnia* strains and species (see Fig. 3) to determine if it was common to the population of interest (Figs. 6A, 7A). A suggestion of how variable the marker was in the population was indicated by the presence or absence of the marker in isolates known to differ morphologically from ***S. parasitica.***

2 . SSCP

Once an appropriate primer-pair was identified, the genomic fragment it amplifies was targeted for base sequencing. However, dealing with even a modest sized population of fungi and markers with approximately 300 base pairs could make the sequencing aspect an overwhelming task. Burt et al. (1994), therefore, inserted an intermediate step that permits screening for

mutations in the selected markers. This process depends on separating the DNA duplex into its two complementary strands. Each single strand takes on its own unique shape that will result in differing mobilities when run on a gel of proper construction. This technique, called single-strand conformational polymorphism (SSCP) is well established, and is capable of identifying single base differences.

The major steps in our SSCP protocol are listed below:

a. Choose marker common to all members of the population.

b. Run cleaning gel and transfer marker to water.

c. Reamplify in presence of ^{35}S ATP to label the DNA.

d. DNA is heated in "stop solution" containing foramide to produce single strands.

e. The DNA is run on MDE polyacrilamide gels (6-8 hrs).

f. The gel is dried and x-ray film exposed.

3. DNA sequencing

The SSCP step indicates which fungal isolates have differences in their markers. The nature of this variation can then be determined by sequencing representative markers from the population. This step was made feasible by the use of primer-pairs which yielded different ends to the fragment. Sequencing followed the standard Sanger sequencing protocol.

F. 18S rRNA Gene

Primers NS 1 and NS 8 were used to amplify a relatively large section of the gene, that was subsequently cleaned, and then sub-divided with other universal primer sets. Asymmetric amplification was followed by cleaning through Millipore (30,000 NMWL) filter units. The products of Sanger sequencing with ^{35}S ATP were electrophoresed on acrilamide gels. The dried gels were then used to expose x-ray film. Sequence was read on a Biorad gel reader. When feasible the sequence was read and checked in both directions,

IV. RESULTS

A . RAPD's

In the first part of this study a total of 527 bright bands were scored from the 16 isolates examined (Fig. 2). DNA extracted from 4 separate cultures of isolates 292- 1 and 170-3 produced identical banding patterns. Repetitive analyses of these two isolates also allowed us to define an error term for comparison between gels and between multiple readers. The level of error in this study was ± 2.5 %, therefore, bands between replicated gels were considered to be the same if they differed by less than ± 2.5%. The inclusion of the reference template DNA's in each amplification and each gel permitted the direct comparison of results. On this basis we were also able to reject some of the results. The rejected results were repeated and when they confirmed the original results (most often) were accepted. When the repeats did not confum the original results the analyses were repeated until uniform results were obtained.

In the first part of the study there were several very bright, reproducible bands that were specific for S. ***parasitica.*** The results of the Jaccard's Coefficient analysis are presented visually in a clock diagram (Fig. 5) and the UPGMA dendrogram is presented in Figure 3. Approximately 12% of the bands were present in all isolates studied. Those isolates showing- least similarity (<30% bauds in common) are distinguishable as different morphological species. These isolates are Isolate ***379 (S. hypogyna),*** Isolate 36144 (S. ***diclina),*** Isolate 53 (S. *ferax*), a small cluster; Isolate ***09 (S. diclina)*** and Isolate 36 (not identifiable with morphology), and a large cluster containing the rest of the isolates (S. ***parasitica).*** Isolate 09 has diclinous antheridial hyphae, centric oospores and short single hooks on the secondary zoospore cyst (Mueller and Whisler, 1994). It was expected to cluster with Isolate 36144, S. ***diclina,*** from England, but it did not. Isolate 36 does not reproduce sexually nor does it produce recognizable secondary zoospore cysts thus making it morphologically indeterminable. The group that represents S. ***parasitica*** maintains its integrity to the 30% bands in common level, where five groups separate out and maintain their integrity to the 50% level. The five groups are: Isolates 292-1, 288, 286-1, 285-1, 261-1, collected from adult chinook salmon at the time of spawning; Isolates 36147 and "Beakes" isolated from salmonids collected from Lake Windermere, England; Isolate 291 from adult chinook salmon at the same time and location as isolates 292-1, 288, 286-2, and 285-l; Isolate 119-l from adult rainbow trout, causing an epizootic; Isolates 170-3 and 174-1, from juvenile chinook salmon. The two isolates from England clustered together within the ***S. parasitica*** group showing greatest affinity for 29 l-l. Above the 70% level all isolates were separate having no bands in common, this is believed to represent a combination of individual variation and random noise.

In the second part of our RAPD survey of fungi from the Columbia River Basin, we selected primers that had been particularly useful in the first screening, and used them to look at a broader sample of our local isolates (Table 2, Fig 5A). Another population of interest was related to an epizootic of spring chinook juveniles at the Hood River Hatchery. In this study a total of 146 bands were scored, generated from 14 primers and primer pairs (Fig. 5B). The isolates from Hood Canal grouped together at the 44% level and remained grouped until the 95% level. Four isolates were not distinguishable from one another, grouping at the 100% level. Two additional isolates joined this group at the 98% level. A second group of 3 isolates joining the first at 95% are not as tightly clustered as the first group.

B. SWAPP

1. Selection of small paired primers

The initial screening of all possible combinations of 10-mer primers from Operon sets C, D & E provided 30 pairs that potentially possessed the right characteristics of size, brightness and uniqueness. The most promising are listed in Table 3. The primer-pairs were then run against our selected set of *Saprolegnia* isolates (Table 1) to see if the DNA fragment they amplified was found in all members of the population.

Primer-pair El/E3 (Fig. 6A) and E5/15 (Fig. 7A) illustrate two different types of marker distribution. In the case of El/E3 the amplified DNA fragment was present in essentially all strains of S. *parasitica*, (as previously determined by other, morphological criteria), but the marker was not found in more distantly related species (Isolates 379, 36, and 9).

The marker therefore, has the potential of selecting for S. ***parasiticu.*** Its presence is clearly more variable than the marker amplified by primer-pair E5/E15. This pair is one of the very few that yielded markers common to all tested isolates (Fig. 7A). This conservative aspect predicted that we would find little variation in that marker at the sub-specific level of a S. ***parasitica*** population when we progressed to SSCP gels.

2 . SSCP

These ideas concerning relative distribution were tested by comparing El/E3 and E5/E15 markers with the SSCP procedure. As Figure 8 illustrates, El/E3 shows distinct differences between isolates 291 and 174.

In contrast E5/E15 displays little variation as predicted in the previous RAPD screening (Fig. 7). Some gels were also run at cooler temperatures, as produced by continuous fans, to see if band migration varied at different temperatures. No differences from the standard temperature were noted.

The SSCP procedure did serve its role in screening groups of fungal isolates for marker differences, but we found the technique difficult to standardize.

3. DNA sequencing

Following SSCP screening, representative markers with different band patterns were prepared for Sanger deoxy sequencing. At this point we encountered a major obstacle in the SWAPP strategy. Distinct sequence patterns were not achieved. Consultation with the Taylor lab indicated they had experienced the same problem. Their solution was to go to larger primers (i.e. from 10- to 20-mer primers). Having invested so much effort in identifying our primer-pairs, it was recommended that we lengthen our 10-mer primers by adding ten arbitrarily selected bases to the outside of the existing primers. These extended primers could then be used in the SWAPP secondary amplification step (i.e. after initial amplification with the 10-mer primer).

These extended primers permitted sequencing of two E1/E3 markers that displayed different mobilities in their SSCP screening (Fig. 8). The sequence of these genomic markers is presented below.

TABLE 1

SELECTED ISOLATES

First RAPD Trial

Species	Site	Source	Isolate #
S. parasitica	Cowlitz Hatchery, WA	Adult Chinook	292-1
C1	"	"	288
c 2	"	"	286
c 3	"	"	286
c 4	"	"	285
c 5	"	"	291
E	Entiat Hatchery, WA	"	261
W1	Lake Windermere, U.K.	Char	ATCC 36147
w 2	"	Brown Trout	TP41 (?)
L	Leeburg Hatchery, OR	Adult Rainbow	119
H1	Hood Canal Hatchery, WA	Juvenile Chinook	174
H2	"	"	170
S. hypogyna			
HY	University of Washington Hatchery Pond, WA	Pond water	379
s. sp.			
sp.	Hupp Springs, WA	Juvenile Chinook	36
S. ferax			
FE	Stevens Pass, WA	Pond water	53
S. diclina			
DI	University of Washington Hatchery, WA	Adult Rainbow	9
DW	Lake Windermere, U.K.	Lake water	ATCC 36144

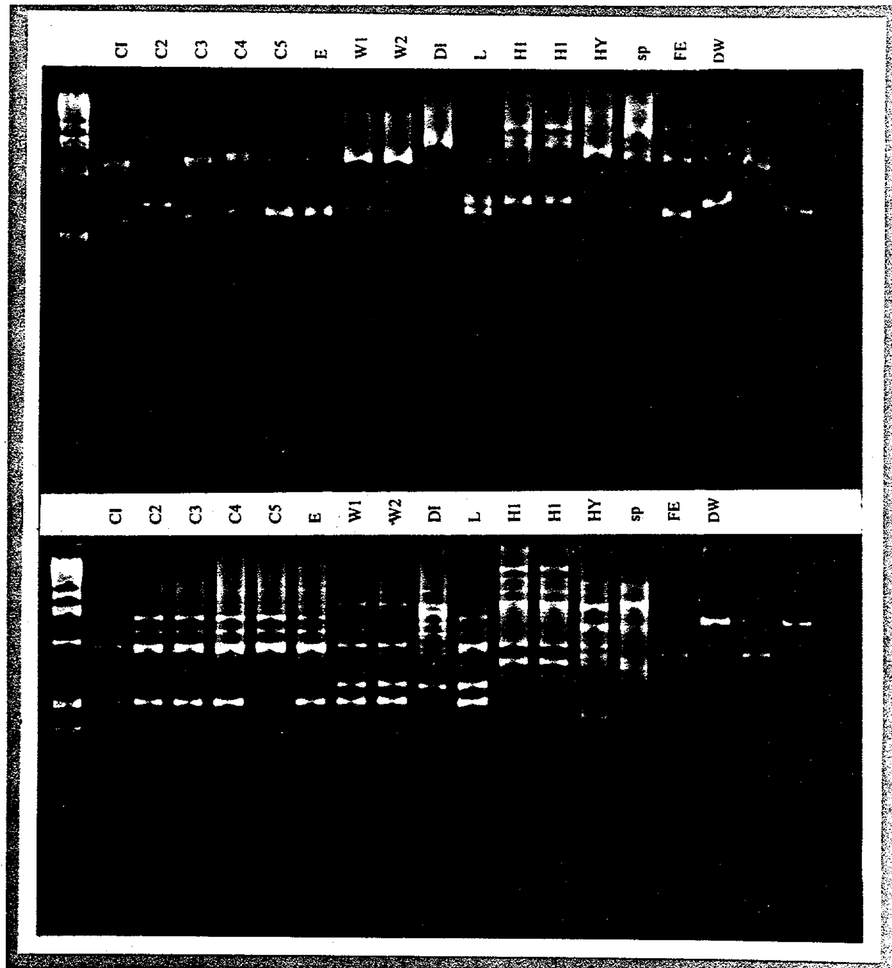


Figure 2.

Two RAPD gels that illustrate how the isolates in Table 1 were compared to each other. The relative size or position of the marker may be estimated by comparison to the 1 kb ladder at the left of the lanes. The upper gel employed the Operon primer E 1; the lower the primer E 3.

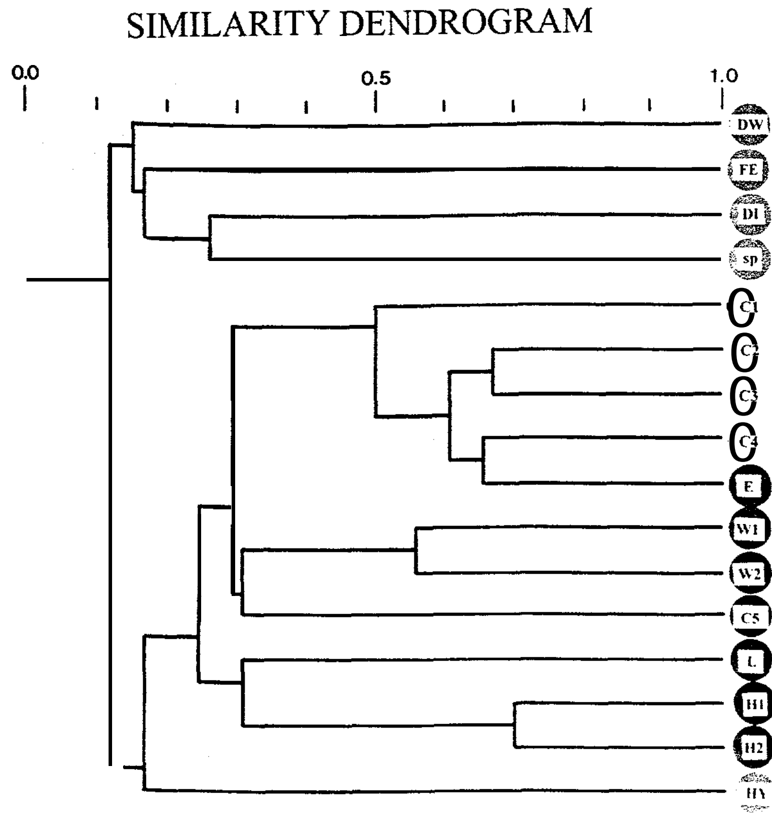


Figure 3.

Dendrogram indicating the relative similarity of the isolates in Table 1. A total of 527 bands or different markers were scored in this study. Isolate relationship was determined on all isolate pairs using Jaccard's Coefficient of Similarity (percent band in common) and analysis based on UPGMA (unweighted pair-group arithmetic average). The dendrogram was re-drafted as similarity.

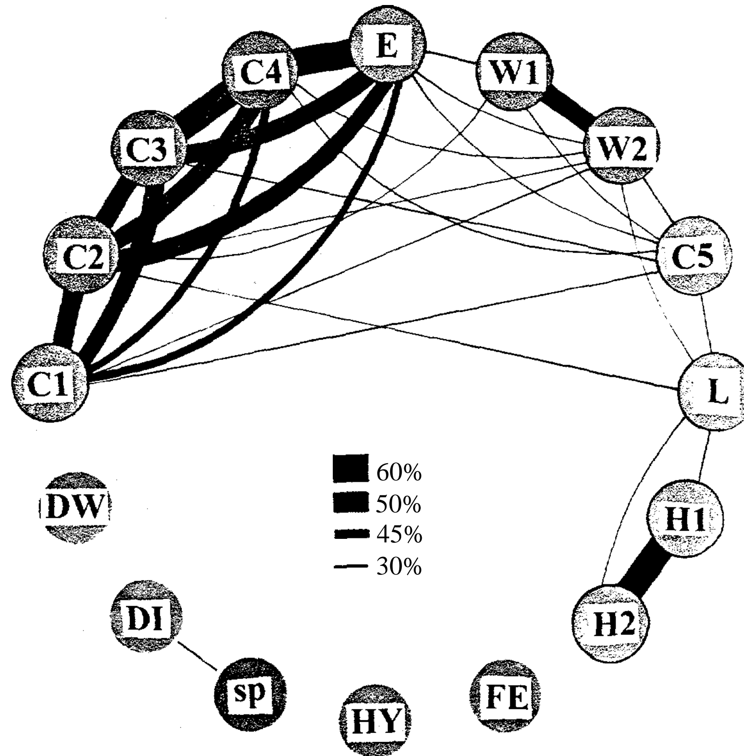


Figure 4.

Percent similarity of the isolates listed in Table 1. The information presented in the dendrogram (Fig. 3) has been re-drafted to show similarity groups of the different strains of *Saprolegnia.* These results suggest a high degree of similarity for most isolates of S. *parasitica* from the Columbia River Basin, and significant dissimilarity from other species of the genus, including S. *diclina.*

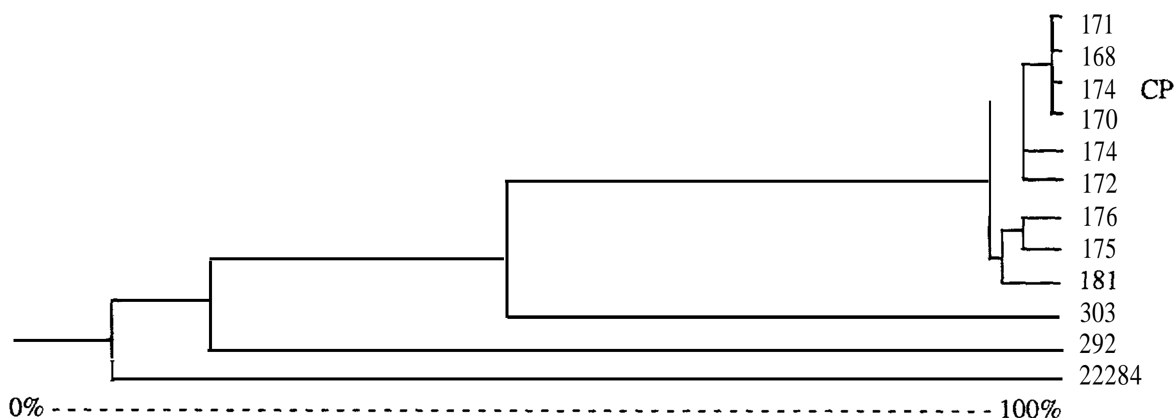
TABLE 2

INDEX TO ISOLATES OF *SAPROLEGNIA* TESTED WITH RAPD'S

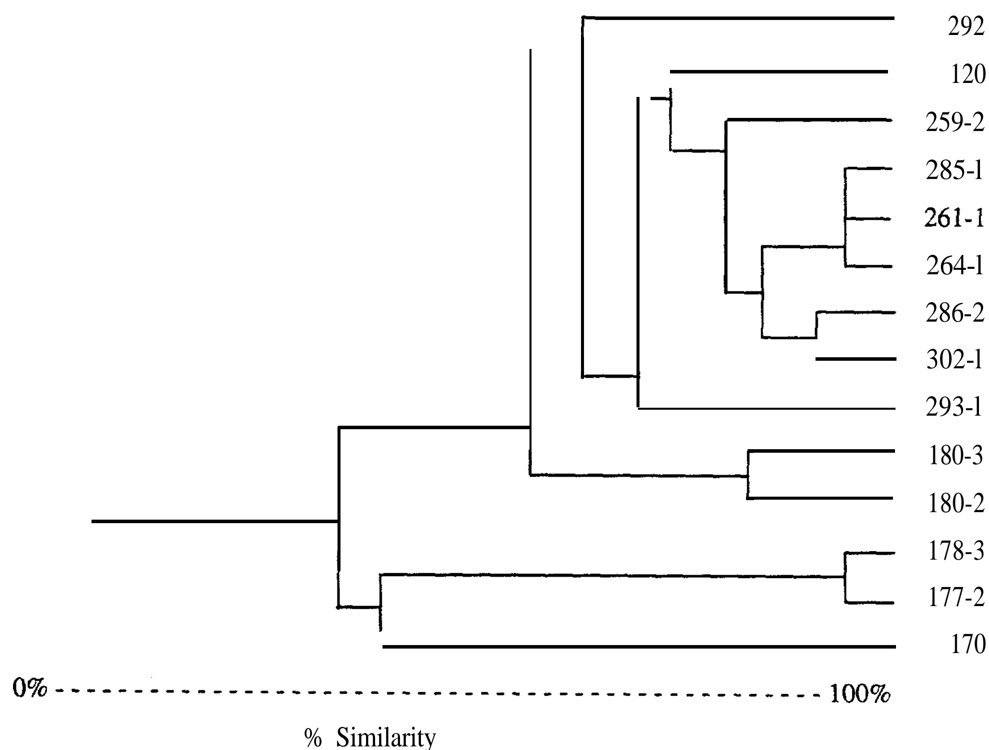
Isolate Number	Geographic Locality	Source (Species/lesion location	age)	Growth Rate (mm/24 hr)	Sexual Reprod.
9	Seattle (Hatch), WA	Rainbow/Flank	adult		yes
36	Hupp Spring (RP), WA	Chinook/Internal	juvenile	16.5	no
48-3	Similkameen (RP), WA	Chinook/Nare	juvenile	16.4	no
95-32	ID				
106-2	Santiam (Hatch), OR	Chinook/Dorsal Fin	adult	15.9	yes
112-2	Santiam (Hatch), OR	Chinook/Caud. Fin	adult	14.6	yes
113-1	Santiam (Hatch), OR	Chinook/Flank Flap	adult	11.4	yes
119-1	Leeburg (Hatch), OR	Rainbow/Pect. Fin	adult	13.4	no
119-2	Leeburg (Hatch), OR	Rainbow/Pect. Fin	adult	12.5	no
120-1	Leeburg (Hatch), OR	Rainbow/Gill	adult	17.1	no
121-3	Leeburg (Hatch), OR	Rainbow/Gill	adult	14.4	no
123-2	Leeburg (Hatch), OR	Rainbow/Gill	adult	16.8	no
130-2	Santiam (Hatch), OR	Chinook/Caud. Fin	adult	9.9	no
135-2	Dexter (RP), OR	Chinook/Head	adult	15.5	yes
136-1	Dexter (RP), OR	Chinook/Dorsal Fin	adult	14.0	no
143-1	Dexter (RP), OR	Chinook/Gill	adult	11.6	yes
147-3	Dexter (RP), OR	Chinook/Gill	adult	16.0	yes
148-2	Dexter (RP), OR	Chinook/Pect. Fin	adult	15.0	yes
149-2	Dexter (RP), OR	Chinook/Gill	adult	15.9	yes
149-3	Dexter (RP), OR	Chinook/Gill	adult	16.4	yes
170-3	Hood Canal (Hatch), WA	Chinook/Dorsal Fin	juvenile	8.0	no
174-1	Hood Canal (Hatch), WA	Chinook/Caud. Fin	juvenile	12.3	no
177-2	Hood Canal (Hatch), WA	Chinook/Pect. Fin	juvenile	13.8	no
178-3	Hood Canal (Hatch), WA	Chinook/ Caud. Fin	juvenile	13.6	no
180-2	Hood Canal (Hatch), WA	Chinook/ Pect. Fin	juvenile	10.4	no
180-3	Hood Canal (Hatch), WA	Chinook/ Pect. Fin	juvenile	15.8	no
259-2	Entiat (Hatch), WA	Chinook/Caud. Fin	adult	14.1	yes
259-3	Entiat (Hatch), WA	Chinook/Caud. Fin	adult	14.1	yes
261-1	Entiat (Hatch), WA	Chinook/Gill	adult	14.3	yes
264-1	Entiat (Hatch), WA	Chinook/Gill	adult	13.8	yes
285-1	Cowlitt (Hatch), WA	Chinook/Eye surface	adult	14.8	yes
286-1	Cowlitz (Hatch), WA	Chinook/Operculum	adult	15.2	yes
286-2	Cowlitz (Hatch), WA	Chinook/ Operculum	adult	14.5	no
288	Cowlitz (Hatch), WA	Chinook/Snout	adult	14.7	yes
291-1	Cowlitz (Hatch), WA	Chinook/ Peduncle	adult	13.7	no
292-2	Cowlitz (Hatch), WA	Chinook/ Operculum	adult	14.2	yes
293-1	Cowlitz (Hatch), WA	Chinook./ Caud. Fin	adult	15.5	no
302-1	Cowlitz (Hatch), WA	Chinook/ Flank	adult	13.0	yes
303	Silkeborg, Denmark	Rainbow/?	adult	12.0	no
379	Seattle, WA	Water Sample		12.2	yes
39209	British Columbia, Canada	Sockeye/?	adult	15.9	no
36144	England	Water Sample		17.6	yes
36147	England	Arctic Char/?	adult	10.6	no
"Beakes"	England				yes

Figure 5.

Dendrograms of Similarity. Fig. 5A. Percent similarity of isolates of S. ***parasitica*** from an epizootic of saprolegniasis in juvenile spring chinook salmon at the Hood River Hatchery. The high degree of similarity of the isolates responsible for this outbreak in young fish is in contrast to Fig. 5B, a second evaluation of relatedness of isolates, mainly from adult salmon, from throughout the Columbia River Basin.



5A. Hood River Hatchery epizootic.



5B. Second comparison of isolates of ***S. parasitica*** from the Columbia River Basin.

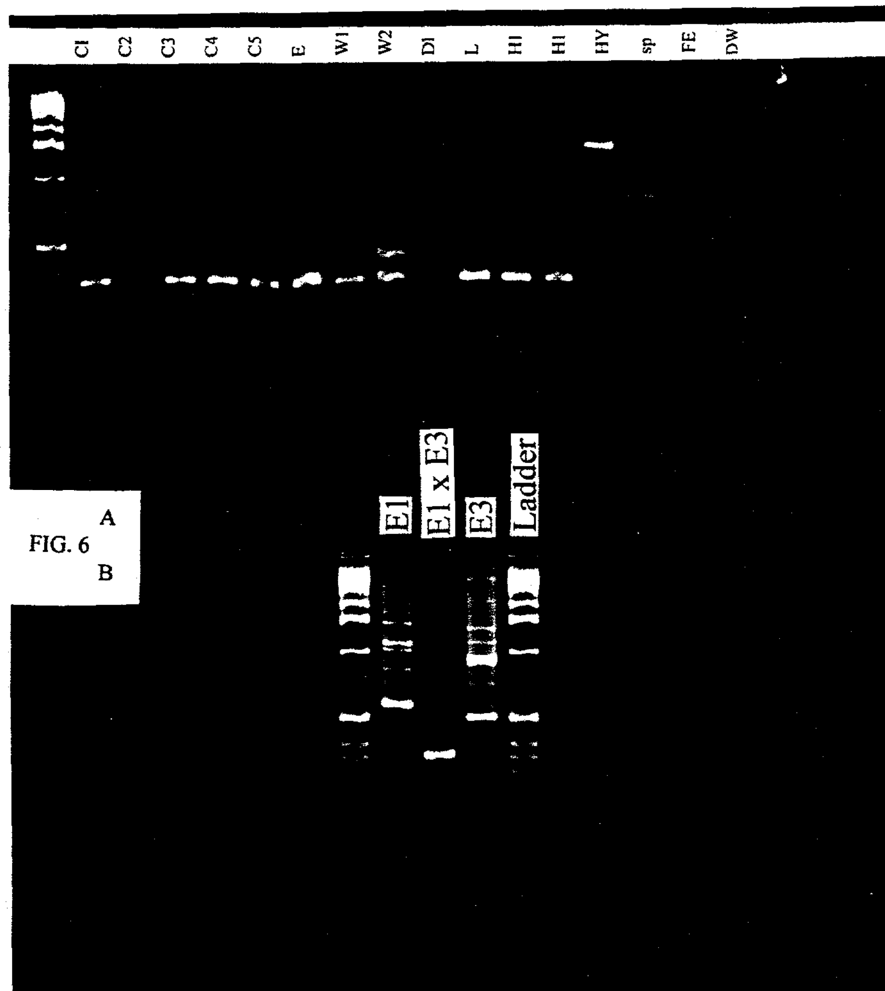


Figure 6.

Primer pair E1/E3. Figure 6B illustrates a marker selected by the two Operon primers El and E3. The marker is relatively small as indicated by the 1 kb ladder, it is relatively bright, and its position is different from either of the single primers. Based on the information from RAPD analysis the marker is common to most ***S. parasitica*** isolates but not to other *Saprolegnia* species (Fig. 6A).

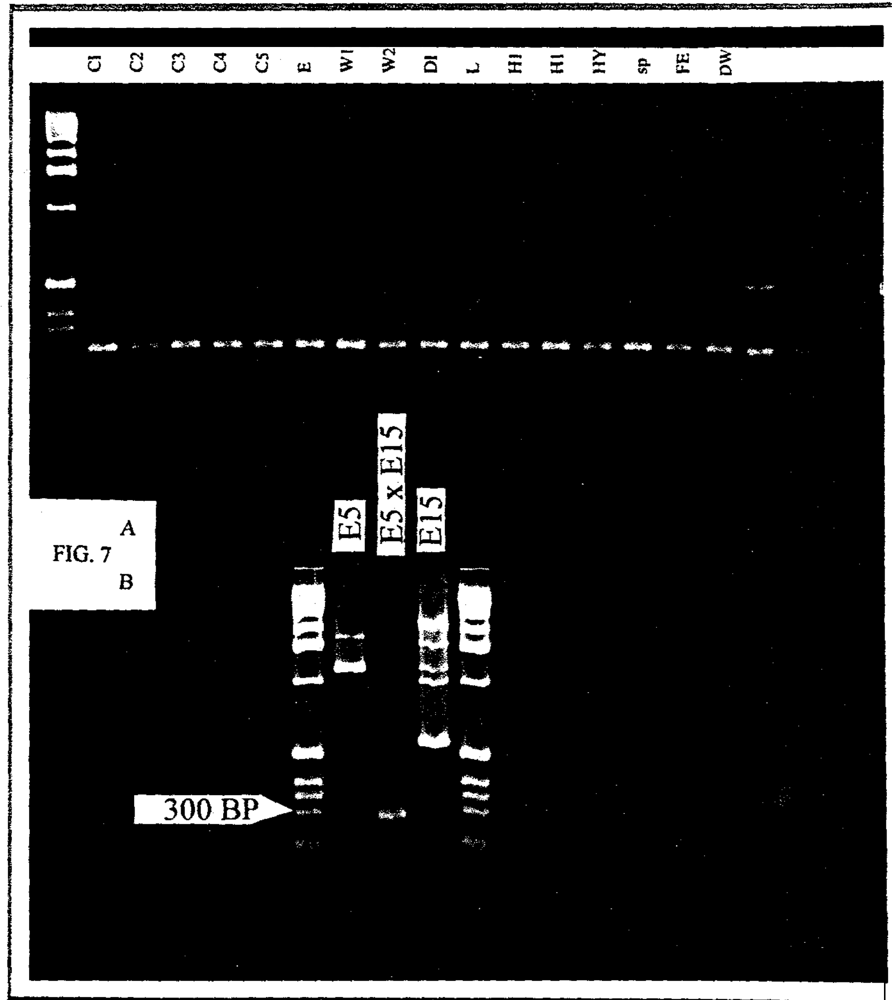


Figure 7.

Gel illustrating marker E5/E15. In contrast to El/E3 (Fig. 6), this primer pair (Fig. 7B) illustrates one of the few conservative markers that was found to be common to all isolates and species in the selected set (Fig. 7A). Little variation is also seen in the SSCP gel (Fig. 9).

TABLE 3

PRIMER-PAIRS THAT DISPLAY "SWAPP" POTENTIAL

(i.e. select small, robust, and distinctive markers)

	Pair	Brightness	# Markers	Size (bp)	Select for *S. parasitica?*
1.	El/E3	xxx	1	344	yes
2.	E5/E15	xxx	1	300	no
3.	D6/D15	xxx	2	300	yes
4.	D5/D10	x	1	344	no
5.	D8/D15	xx	2	344	yes
6.	C9/C19	xx	2	460	yes
7.	C13/C18	xx	2	396	yes
8.	C14/C16	xx	2	460	yes

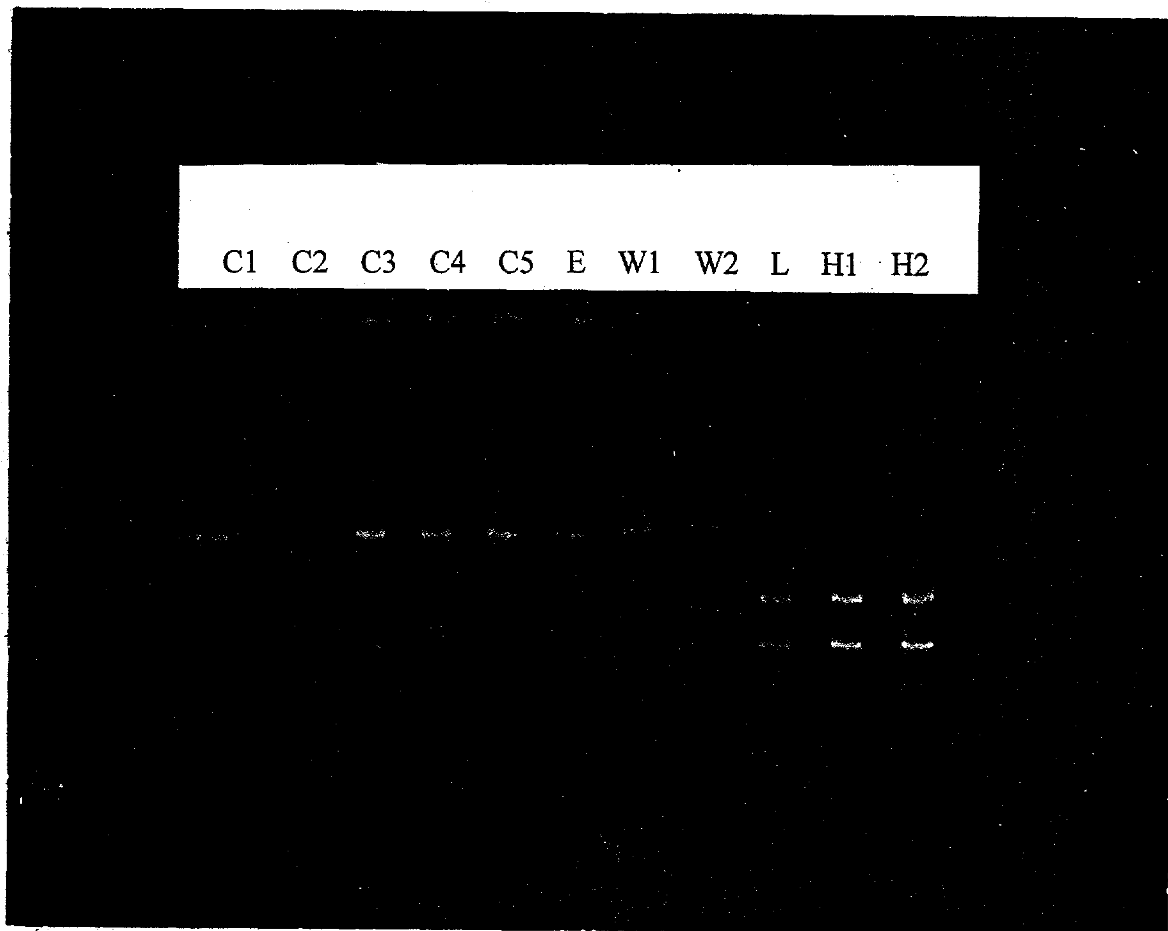


Figure 8.

SSCP with E1/E3. The results of this gel indicate that there is significant variation at the sub-specific level. Since members of the Saprolegniales are diploid, each marker now appears as two bars, one for each strand of DNA. Several isolates from the Cowlitz Hatchery (Cl, C3, C4, C5) and Entiat 1 appear identical and homozygous. Strains H l and H2 are different from the preceding but are also homozygous. Strains C2, W 1, W2 and L are interpreted as being different, but heterozygous with respect to this marker.

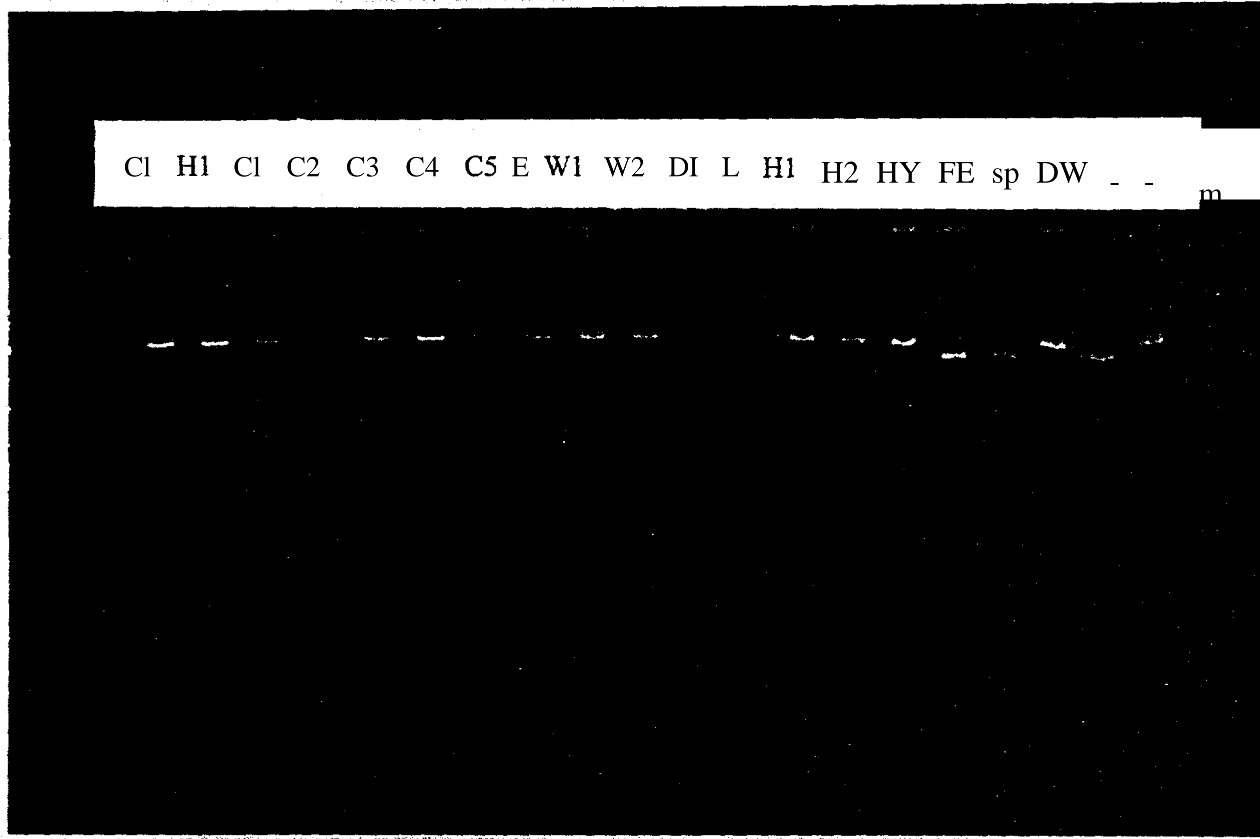


Figure 9.

SSCP with E5/E15. Very little variation between isolates is seen in this gel.

It will be noted that the marked difference between isolates 292-l and 174-1 as seen on the SSCP gel (Fig. 8) is related to two transitions (G-C) in position. Based on the SSCP pattern, isolates 292-1, 285, 286 and 261 and are expected to have the same sequence and 174-l and 170-*3* are the same.

C. 18S rRNA Gene Sequence

The sequence obtained is presented in Appendix II. An area of approximately 300 base pairs between NS 1 and NS 2 was sequenced and read in both directions. This is a relatively "informative" portion of the gene and this sequence should help in relating Isolate 29 1- 1 to other water molds. The universal primers did not provide sequence in both directions in some areas of the gene.

V. DISCUSSION

The first study suggests that the species of ***S. parasitica*** reported from fish can be separated using RAPD analysis. There is a break of about 12% bands in common (between 20% and 30% bands in common) which separates the isolates into several groups (Figs. 3, 4). Above 30 bands in common the degree of relatedness of the largest group is shows no large breaks until 70% bands in common, the highest value found. This division between 20% and 30% bands in common agrees with species level divisions based on morphology. This analysis supports the separation of *Saprolegnia* into the species *ferax*, ***hypogyna,*** and ***diclina.*** Further, this analysis supports the separation of the poorly defined species ***S. parasitica*** from the other species reported from salmon lesions. The use of the long bundles of hooks on secondary zoospore cysts does appear to be a valid morphological character as suggested by others. These isolates, recognized by RAPD analysis, are representative of the vast majority of the isolates cultured from surface fungal lesions of salmonids in this study, suggesting again that there is a strong pathological relationship between this fungus and its host.

The ***S. parasitica*** isolates ("Beakes" and 36147) from England are separated from the rest of the isolates by no greater a distance than the distance between isolates from the Columbia River Basin. The isolate 36144 (England) determined to be a *S. diclina* on the basis of its morphology is related to isolate 9 (Washington) also determined to ***be*** a ***S. diclina only*** on the basis of 18% bands in common. These two isolates 36144 and 9 as separated by RAPD analysis suggest that they are separate, although morphologically similar, species. Isolate 36 is an unidentifiable non-sexually reproducing isolate, found growing internally in a chinook salmon fingerling, which would have been identified as a S. *ferax* on the basis of its growing habits, [based on the description of the growing habits of S. ***invader-is*** (Davis and Lazar, 1940) later declared a synonym of S. *ferax* by Seymour (1970)]. Isolate 36 on the basis of its RAPD analysis is shown to be closely related to Isolate 9, a ***S. diclina.***

Our analysis of the ***S. parasitica*** group from the Columbia River and its tributaries suggests that, at a minimum, three separate populations are present. The largest grouping contains isolate 261-1 from Entiat Hatchery located 450 miles farther up the Columbia River from the Cowlitz River on which the Cowlitz Hatchery is located and from where the rest of the isolates originated. Isolate 291 also from Cowlitz Hatchery is more closely related to the samples from England (Fig. 5) than it is to those from Cowlitz. The pattern can be explained by the fact that these are adult

chinook salmon that are infected in the river before returning to the hatchery from which they were sampled.

Isolate 119- 1 from Leeburg Hatchery on the McKenzie River and the two -isolates (170-3, 174-1) from the geographically separated Hood Canal Hatchery are related to each other. The Hood Canal isolates are separated from the other isolates *in* the ***S. parasitica*** group. The Hood Canal isolates are related to the rest of the group only through isolate 119. Further the Hood Canal isolates are less like the main group of ***S. parasitica*** than to the isolates from England.

The isolates from England, 36147 and "Beakes", have many inter-relationships above the 30% level and are clearly situated in the cluster of ***S. parasitica*** (Fig 4).

Segregating the populations of ***S. parasitica*** into separate species might be considered since the level of bands in common is quite low. However it seems unlikely that three morphologically similar species with the same habitat specific requirements would inhabit the same area. Further there is no other break in the pattern of bands in common as determined in this study. The high level of individual variation comprising at least 25% of the total number of the bands in common (Fig. 3) suggests that genetic variability in this species is very high. If the individual variability is removed from the data then the bands *in* common for the S. *parasitica* group approaches 50% and the gap between the species becomes almost 15% or more.

The second study was established to extend the results of the first study to many more isolates, attempting to understand more completely the geographical distribution and the total number of populations present in the Columbia River Basin (Fig. 5). The results reinforced the idea of several different sub-populations of *S.* ***parasitica*** in the Columbia River.

The results of the second study has also provided information on the origin and structure of the S. ***parasitica*** population involved in one outbreak of saprolegniasis in juvenile chinook (Fig. 5A). Isolates 168, 170, 171, 172, 174, 175, 176, and 181 were involved in a single out break of saprolegniasis which was reported resistant to the 30% seawater normally used for treatment. This group clustered together at the 95% level representing a single population as might be expected from the limited geographical area encompassed by the freshwater source of the hatchery. The isolates split into two groups at the 95% level with a total of five subdivisions. Four of the isolates grouped together at 100% suggesting that they were derived from a single point source. The genetic similarity of this population suggests clonal development of the fungus.

In our sample set, the primer-pair El/E3 appears to be selective for strains of ***S. parasitica*** on salmon. If this proves to be the case, it has the potential for being a useful probe for pathogenic strains of ***Saprolegnia.*** Since we know the 300 bp sequence of the El/E3 marker, this information could be used to construct either a radioactive or fluorescent diagnostic probe. Such a probe could conceivably be useful in hatchery sanitation efforts and fungal distribution studies.

As more SWAPP markers are sequenced, it will be possible to use this sequence information to construct phylogenetic trees that are more definitive than the information we have gained with RAPD's and the UPGMA analysis method.

When we encountered the 10-mer sequencing problem we made a decision to put more emphasis on direct RAPD analysis and less in the SWAPP strategy. This was based on the considerable labor involved in the latter approach and a reduced emphasis on the question of

clonal versus sexual reproduction. As will be developed in a later section, we now suspect that S. *parasitica* may not be as weakly sexual as the literature has indicated.

PATHOGENICITY

I. INTRODUCTION

These studies are preliminary and were conducted to assess methods of pathogenicity testing. Saprolegniasis of adult chinook salmon in the Columbia River Basin is associated with a variety of populations and strains of *Saprolegnia parasitica* as shown in the first part of this report. The existence of numerous populations lends some support to the anecdotal reports of saprolegniasis pathogenicity varying among outbreaks. In the past it has not been possible to associate species, populations or strains of *Saprolegnia* with outbreak severity in adult chinook salmon. The adult salmon commonly become infected before they enter the hatchery. The Columbia River turbulence mixes the various populations or strains and, as a result, fish sampled from a single locality commonly have more than one strain. For example, Cowlitz Hatchery was sampled extensively and during one sampling period several populations were found within the group of fish sampled. Thus it is not possible to determine if deaths are related to a particular strain of *S. parasiticu.* Likewise it is not possible to relate treatment success or failure to mixed *S. parasitica* populations. Examination of the physiological data (Mueller and Whisler, 1994) suggests that the slow growing isolates of *S. parasitica* are eliminated from the lesions over time. The slow growing strains may simply be out competed in the wound or the formalin treatment may be most effective for the slow growing strains, both again suggesting that different populations or strains are present. These results all suggest pathogenicity might differ between isolates, strains, or populations.

To address the pathogenicity problem in a direct manner a preliminary set of experiments were conducted. These preliminary experiments were carried out to examine the feasibility of two differing methods of pathogenicity testing. The first method based on the "ami-momi" (shaking in a net) treatment (Hatai and Hoshai, 1993, 1994). The "ami-momi" treatment is based on, but is in excess of, treatment that might be received by a fish during handling in a hatchery environment. The second method using social stress is also based on conditions that are present in hatcheries or in the natural environment (DeWald and Wilzbach, 1992; Pickering, 1993).

The direct tests described above were also accompanied by a set of exploratory trials in biological control, and efforts to monitor the pathogen in its aquatic habitat.

II. MATERIALS AND METHODS

A. "Ami-momi" Treatment

Fifty chinook salmon yearlings surplus to other studies where made available for the "arm-momi" treatment experiments. The fish were divided up into five lots of eight fish, three lots receiving "ami-momi" and *S. parasitica,* one lot receiving "ami-momi" treatment and no pathogen and one lot held as a normal control. The fish were kept at approximately 8°C using untreated water pumped from Lake Washington. The "ami-momi" pathogen treated fish received 40,000 zoospores/l acclimated to 20°C, 4,000 zoospores/l acclimated to 10°C and 40,000/l zoospores/l

acclimated to 10°C. The non-infected fish from the first experiment plus replacement fish from the controls were treated again in a second experiment using 4,000 zoospores/l,400 zoospores /l, and 40 zoospores/l, all acclimated to 10°C. The controls had reduced numbers of fish. The non-infected fish from the second experiment were exposed a third tune to concentrations of S. ***parasitica*** of 4,000 zoospores/l, 400 zoospores/l, and 40 zoospores/l, all acclimated to 10°C and only a "ami-momi" control was established. The main stock tank was used as the normal control. For all experiments water flow through the tanks was stopped and oxygen requirements were met with air bubblers. The water volume was reduced to 100 liters and the zoospores added. The fish were held in the zoospore suspension for six hours in the first two experiments and for 24 hours in the last experiment. After the exposure period the water flow was re-established and continued throughout the rest of the experiment. "Ami-momi" treatment was given for two minutes with the fish in a single group in the first experiment, and for one minute with two fish treated at a time in the second experiment. The thud experiment treated fish for two minutes with two fish treated at a time

Isolate 292-l was selected as the initial fungal isolate to test. Four small pieces of the leading edge of a cm agar culture were cut out with a cork borer. These four pieces were transferred to 4 one liter flasks containing 500 mls of GY media (Hatai and Hoshai, 1993). The isolates were incubated for 4 days at 20°C in a rotary shaker. The mycelium was drained, rinsed three times with sterile lake water and weighted. Wet weights averaged 30 grams. The mycelium from each flask was placed into 1 liter of sterile lake water and held at the acclimation temperature. After 5 to 6 days the concentration of active zoospores reached 4,000 to 5,000 per ml. The experiment was started when sufficient numbers of zoospores were available.

Zoospore counts were made with the following method. A one and one half ml sample was removed from the rearing container after gentle agitation. Four µl of fluorescein diacetate (0.1% in acetone) and 10 µl of nickel sulfate (1 .O% $NiSO_4 \cdot 6H_2O$) were added to the sample. The sample was inverted once and drawn up into a pasteur pipette and immediately added to a Sedgewick-Rafter Cell for counting (Willoughby and Pickering, 1987; Soderstrom, 1977). The counts were made at an excitation wave length of 450-490 nm with a 520 nm barrier filter with a Zeiss epiflourescence microscope. Lesion size expressed as a percent of body surface was determined by preparing a grid of 225 squares on a photocopy transparency. The overall dimensions of the grid were varied with the size of the fish, so that the length of the grid equaled that of the fish. An outline of the fish was traced on the transparency and then the area of each lesions was cross hatched. The transparency was photocopied and the fish outline was cut out and weighed. The lesions were cut out from the fish out line and weighed. The weights of the outline and the lesions were compared.

B. Social Stress

For the social stress experiments, steelhead trout (0. *mykiss*) were available in sufficient quantities and time was available for two experiments. In the first, a single or pair of fish were placed in baskets of approximately one liter in volume, the baskets were 3/4 submerged, four at a time in a larger tank. The individual fish in each pair were taken from separate holding or isolation tanks. The screen covered sides of the basket had sufficient open area to permit water exchange with the tank where water flow was maintained. The position within the tank was fixed, with the first position nearest the inlet. In the top of the basket a cylindric cage of window screen was

inserted to hold a hemp seed that was colonized with *S. parasitica* isolate 292-l. A new seed was added to the seed already present on alternate days. The experimental design included ten baskets in which unpaired control fish were placed, ten baskets containing pairs of fish with fins in perfect condition having been held in isolation for several weeks, with one fish 8-10% longer than the other; and 28 baskets in which pairs of fishes were selected from separate holding tanks and which had fins with varying degrees of erosion. For the 28 baskets, 28 fish were selected in size with a fork length (FL) that ranged between 91 and 97 mm. Fourteen fish were then selected that were 10% smaller, ranging in size from 81-86 mm FL and paired with the appropriate fish. Fourteen fish were also selected that were 10% larger ranging in size from 98 to 106 mm FL and paired with an appropriate fish. In this way an internal control was established to determine if fish size was a factor in infection.

Colonized hemp seeds were prepared by boiling the hemp seed until the coat split open and the radicle was exposed (Emerson, 1958). The seed was then placed at the growing edge of a *Saprolegnia* colony growing on cornmeal agar held at 10°C. After 48 hours the seeds could be seen to be well colonized. The seeds were carefully removed from the agar surface and placed into the window screen containers inserted into the tops of the baskets containing the fish. The seeds were placed in the baskets 24 to 36 hours before the fish. All baskets received the hemp seeds including the baskets containing the individual control fish. Unpublished data indicates that each seed can produce between 100,000 and 250,000 primary zoospores over a 96 hour period. Repeated sampling indicated that 9-10 zoospores per ml were present in the fish baskets during the course of the experiment.

A second experiment was set up using the same methods as the first social stress experiment. This experiment was to determine if the bacterial tail rot (see below) and the saprolegniasis observed in the first experiment were related to each other. In this study half of the experimental fish received hemp seeds colonized with *S. parasitica* and half received non-colonized hemp seeds. All of the experimental fish came from the same holding tank.

Water temperature was monitored and except for a three day drop to 5°C at the beginning of the social stress experiments, the water temperature remained near 8°C.

HI. RESULTS

A. "Ami-momi" Treatment

Infection occurred, in the first experiment, in both the 20°C acclimated and the 10°C acclimated 40,000 zoospores/l tanks indicating that the temperature drop between an incubation temperature of 20°C and 8°C the temperature at which the fish were held, did not seem to affect the ability of *Saprolegnia parasitica* to infect the fish. It was found to be relatively easy to generate zoospores at 10°C and so raising the spores at 20°C was not necessary and therefore not considered again. In both of the 40,000 zoospore/l tanks 25% (2/8) of the fish became infected and were moribund in 6- 12 days with body surface involvement of 23% to 32% (Table 4). One fish was infected (13%) in the 4,000 zoospore/l tank and was moribund after 8 days with 23% of its body surface involved with fungal lesions.

TABLE 4

SUMMARY OF LESIONS

Exp	Conc	Date	DD	jaw	pect f	ventral	pel f	vent	anal	caud p	caud f	adip	dorsal f	dorsal	oper	head	snout	flank	ini p	% sur
#1	40,000	12/7/94	14				x	x	x				xx					XX	5	25
#1	40,000	12/1/94	8		x		x	x	x	xx	xx		xx						7	29
#1	4,000	12/2/94	8		x		x				x	x	xx						5	25
#1	40,000	I1/29/94	6		x		x	x	x	xx		x	xx	x					8	23
#1	40,000	12/5/94	12		x		x		x		xx	x	xx			x	x		8	32
#2	4,000	12/23/94	10		xx	x	x				x	x	xx		x	x			8	29
#2	4,000	12/29/94	16		x	x					x							XX	4	34
#3	40,000	1/14/95	8	x	x		x		x	xx	xx	x	xx		x	x	x	XX	12	35
#3	40,000	1/15/95	9		xx		x		x	x	x	x	xx		x	x	x		10	32
#3	400	2/18/95	12		x		x		x		xx		xx	x	x	x	x		8	33
#3	40,000	2/18/95	12		x						xx	x	xx			x	x		6	36
#3	40,000	2/21/95	15						x		x	x	x			x	x		7	38
#3	40,000	2/22/95	16							x	xx	x	xx						4	32
#3	4,000	2/18/95	12	x	x		x		x						x	x	x		7	26
#3	40,000	2/18/95	12	x	x	x	x		x	x	x	x	xx		x	x	x		12	33
#3	Control	2/24/95	15										xx						1	27
#3	400	2/28/95	19	x						x	x			x	x			XX	6	35
#3	400	3/10/95	29	x						x	x		x			x	x		6	39
#3	400	3/13/95	32	x						x	x				x	x	x		6	30

x = small lesion   xx = large lesion

DD = days to death; pect f = pectoral fin; ini p = initial points of infection; % sur = percent surface involvement

In the second experiment two fish were infected in the 4,000 zoospores/l tank (Table 4). They became moribund after 10 and 16 days with 29% and 34% respectively of the body surface involved in fungal lesions.

The third experiment resulted in six fish (75%) infected in the 40,000 zoospore/l tank (Table 4). They became moribund after 8 to 16 days with 32% to 38% of the body surface involved with fungal lesions. One fish became infected in the 4,000 zoospores/l tank and was moribund at 12 days with 26% body surface involvement. One fish became infected immediately in the 400 zoospores/l tank and was moribund at 12 days with 33% of its body surface involved in lesions. Three additional fish became infected later in the experiment with the most severe wounds on the caudal peduncle and fin. This distribution suggests that the wounds are the result of social interaction. One fish became infected in the control and was determined to be a S. *parasitica.* The fish this experiment were moribund 8 to 16 days after the experiments started and had 30% to 38% surface involvement.

Necropsy showed that even at the shortest period at which observations were made (8 days) fungal hyphae had penetrated from the dorsal fin, the presumed site of initial infection, to the kidney. The muscle tissue near the fin was soft and clearly necrotic. Muscle tissue intermediate between the fin and the kidney was colorless and not as soft as the surface. The area near the kidney was typically hemolytic with blood cells in various states of hemolysis. The muscle was firm and more normal in color. The lesions originating from dorsal fin infection sites result in the largest surface from which salt loss will occur.

Caudal fin lesions after 8 to 10 days show extensive areas of exposed fin rays and after a few more days the backbone may be exposed. These lesions are ultimately lethal as are the dorsal fin lesions. However blood supply and the cross section of the body, through which osmotic agents could be lost, is much smaller than the area exposed to water in a dorsal fin lesion. Lesions elsewhere involving other fins also do not seem to progress as fast as those of the dorsal fin. Pectoral fin lesions eventually reach the body cavity and grow into the heart and digestive diverticula before death occurs. Fish developing lesions in other areas did not survive long enough in these experiments for other observations to be made.

B. Tbe Interim Infections and the Observation of Sexual Reproduction *In Vivo*

Two of the three fish remaining in the 4,000 zoospores/l tank became infected in the interim between the second and third experiments. The infections began on the caudal peduncle or on the caudal fin, again as the result of social behavior. One of the fish died and became heavily colonized before being discovered. This fish was recovered from the tank and preserved after culturing and observation. Oogonia containing normal oospores were observed in the fungal mat. The identity of the isolates was eventually confirmed by RAPD analysis to be the identical with isolate 292-1, the isolate used in the experiments. When the second fish died, again during a period of non-observation, it was not removed from the tank. Small pieces of the mycelial mat were removed over the subsequent weeks. After 14 days normal oogonia were observed to be present in the small mycelial mat samples. The observations were terminated after 73 days at which time there were many normal oospores still present in oogonia found in the mycelial mat. The hyphae on which the oogonia were formed had decomposed but the oogonia remained intact becoming in a sense deciduous oogonia. Only a few free oospores were observed. The third fish survived even though immersed in a constant environment of infective zoospores of S. *parasitica.*

During the period following the third and final experiment two *Saprolegnia* infections were observed, one on a control tank fish and the other on one of the surviving 4,000 zoospore/l tank fish. One infection occurred on the caudal fin and a second occurred on the flank. These infections spontaneously disappeared after about a week. Other anecdotal reports of such observations suggests that this spontaneous cure is not rare. Unfortunately the incident was over quickly and the infecting *Saprolegnia* was not isolated from these fish. *Saprolegnia* collected at the University of Washington Hatchery from adult rainbow trout, with what were reported to be non-threatening lesions, turned out to be *Saprolegnia diclina*. It is possible that the lesions observed after the experiments had ended were also *Saprolegnia diclina*. *Saprolegnia ferax* was not observed to infect any fish during this part of the study although it was collected from the food and other waste material that accumulated at the bottom of the tank during the experimental period.

C. Social Stress

After four days of exposure eight fish had died of stress associated with establishing the experiment and with the initial interactions. These fish were divided into fish with eroded fins and fish that had fins in good shape. Fins from the dead fish were removed and separately placed in culture. The fish from the eroded fins group showed *S. parasitica* growth 24 hours faster and from more sites than the group with good condition fins, where no growth was observed at 24 hrs (Table 5). Growth indicated that at least the mucous has been colonized but does not necessarily mean that the epidermis is colonized. A group of four fish were added from one of the holding tanks and examined in the same manner as a control. The control group showed no growth of *Saprolegnia* however the control group did show growth of *Mucor*, *Aspergillus*, and *Trichodema* as did the two experimental groups. These fungi are typically cultured from salmon saprolegniasis lesions.

A number of fish died during the first and second week of the experiment apparently from a bacterial tail rot (Table 6). At the end of the second week deaths of fish with *Saprolegnia* were apparent (Table 6). Chi square analysis suggests that the there is less than a 2% probability that the proportion of small fish dying was by chance, that there is less than a 5% probability that the proportion of small fish with tail rot dying was by chance, and that there is less than a 1% probability that the proportion of small fish with saprolegniasis were dying by chance alone. A second finding of the first experiment was that basket position was important (Table 7). Mortality was highest for the fish with tail rot in basket 4 the farthest from the inflow. If the deaths from tail rot are removed from the data then the distribution of mortalities from fish with saprolegniasis is not related to basket position.

The second social stress experiment was set up to determine if the there was a relationship between tail rot and saprolegniasis. Tail rot did not occur in either group of fish in the second social stress experiment. Saprolegniasis occurred and caused limited mortality in both groups of fish. In the experimental group receiving the pathogen *S. parusitica* saprolegniasis was observed only in fish which subsequently died. In the group receiving a non-colonized hemp seed a fatal case of saprolegniasis occurred caused by S. *ferax*. This fish also died. The non-colonized hemp seeds were quickly colonized by S. *ferax* and the experimental fish as a result were exposed to high concentrations of zoospores. The numbers of infected fish were low and the disease occurred in both the large and small fish. Saprolegniasis was the only disease seen in these fish and is likely to be the fatal agent.

TABLE 5

NUMBER OF COLONIZATION SITES OF *SAPROLEGNIA*

FISH WITH ERODED FINS (AFTER 24 HOURS)

FISH	CAUDAL	ADIPOSE	DORSAL	ANAL	PECT. 1	PECT. 2	PELVIC 1	PELVIC 2
1	4	0	1	2	1	1	1	2
2	5	1	1	1	1	0	1	1
3	3	0	2	0	1	1	1	0
4	4	0	1	1	0	1	1	1

FISH WITH FINS IN GOOD CONDITION (AFTER 48 HOURS)

FISH	CAUDAL	ADIPOSE	DORSAL	ANAL	PECT. 1	PECT. 2	PELVIC 1	PELVIC 2
1	2	0	1	0	1	1	0	0
2	2	0	1	1	0	0	0	1
3	3	0	1	1	1	1	1	1
4	4	1	1	1	1	1	1	1

CONTROL FISH (AFTER 48 HOURS)

FISH	CAUDAL	ADIPOSE	DORSAL	ANAL	PECT. 1	PECT. 2	PELVIC 1	PELVIC 2
1	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0

TABLE 6

MORTALITY BY DATE

Basket #	Date	Cause	Fish Size
44	3-01-95	Tail Rot	Smaller
13	3-01-95	Tail Rot	Smaller
15	3-02-95	Tail Rot	Larger
21	3-02-95	Tail Rot	Larger
12	3-02-95	Tail Rot	Smaller
32	3-06-95	Tail Rot	Smaller
20	3-06-95	Tail Rot	Smaller
4	3-06-95	Tail Rot	Smaller
8	3-06-95	Tail Rot	Smaller
28	3-06-95	Tail Rot	Smaller
36	3-07-95	Tail Rot	Smaller
43	3-07-95	Tail Rot	Larger
39	3-07-95	Tail Rot	Larger
11	3-08-95	Saprolegniasis	Larger
10	3-08-95	Saprolegniasis	Larger
41	3-09-95	Saprolegniasis	Smaller
31	3-10-95	Tail Rot	Smaller
18	3-10-95	Saprolegniasis	Smaller
24	3-12-95	Saprolegniasis	Control
29	3-13-95	Saprolegniasis	Control
45	3-13-95	Saprolegniasis	Smaller
9	3-13-95	Saprolegniasis	Smaller
42	3-13-95	Saprolegniasis	Smaller
6	3-13-95	Saprolegniasis	Larger
22	3-13-95	Saprolegniasis	Smaller
23	3-13-95	Tail Rot	Larger
46	3-13-95	Saprolegniasis	Smaller
25	3-15-95	Saprolegniasis	Smaller
14	3-15-95	Saprolegniasis	Smaller

TABLE 7

MORTALITY/SURVIVORSHIP

Social Stress Experiment 1

SAPROLEGNIASIS

		Position 1	Position 2	Position 3	Position 4
Experimental	Small Fish	4	5	3	1
	Large Fish	1	1	1	0
Control		2	0	0	0

TAIL ROT

		Position 1	Position 2	Position 3	Position 4
Experimental	Small Fish	1	1	2	8
	Large Fish	0	2	1	0
Control		0	0	0	0

ALL CAUSES

		Position 1	Position 2	Position 3	Position 4
Experimental	Small Fish	5	6	5	9
	Large Fish	1	3	2	0
Control		2	0	0	0

SURVIVORS

		Position 1	Position 2	Position 3	Position 4
Experimental	Small Fish	3	5	4	1
	Large Fish	8	7	6	11
Control		1	2	4	1

TABLE 8

RELATIVE GROWTH OF SAPROLEGNIA

BETWEEN SITES AND TREATMENTS

Non-Treatment

FINS:	caudal	dorsal	pectl	pect2	pelvl	pelv2	aual	body
Dom Fish 24 hr:								
48 hr:		xx			-			
72 hr:		xxx						
Sub Fish 24 hr:	xxx	xxx	xxx	xx	xxx	xxx	xxx	xx
48 hr:	xxx	xxx	xxx	xxx	xxx	xxx	XXX	xxx
72 hr:	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx

Non-Treatment

FINS:	caudal	dorsal	pectl	pect2	pelvl	pelv2	anal	body
Dom Fish 24 hr:								
48 hr:	x		xx		-			
72 hr:	xx		xxx					
Sub Fish 24 hr:	xx	x	xx		xx	xx	xx	
48 hr:	xxx	xxx	xxx	xx	xxx	xxx	XXX	
72 hr:	xxx	xxx	xxx	xxx	xxx	xxx	xxx	

Treatment

FINS:	caudal	dorsal	pectl	pect2	pelvl	pelv2	anal	body
Dom Fish 24 hr:								
48 hr:					-		X	
72 hr:							xx	
Sub Fish 24 hr:	xx				xx	xx	X	x
48 hr:	xx	xx	xx	xx	xxx	xxx	xxx	xxx
72 hr:	xxx	xxx	xxx	xxx	xxx	xxx	xxx	xxx

-=nogrowth x = slight growth xx = moderate growth xxx = heavy growth

In two fish a persistent saprolegniasis occurred and when cultured was determined to be S. *ferax.* The disease was not fatal during the time that it persisted. If the disease had followed the etiology of the first experiment the two fish would have been expected to have died with in three days. The infected fish, which survived 10 days, had not died by the time the experiment was terminated.

Three pairs of fish were selected from the surviving fish to see *if Saprolegnia* microcolonies are present (Table 8). These selected pairs were cultured in a manner similar to that completed in the first social stress experiment. The mucous of the dominate fish was easily sampled and smeared for microscopic examination. No zoospore cysts nor microcolonies were observed. Mucous was more difficult to remove from the submissive fish and hard to spread out for microscopic examination. Microcolonies were found throughout the mucous removed from the submissive fish.

D. Other Studies Related to Pathogenesis

A number of other pilot studies were initiated during the course of this project. It was hoped that they might lay the ground work for moving from the laboratory to the field, and to explore alternative control methods.

1. Biocontrol

a. Hyperparasitism by *Woronina polycystis* on fish-pathogenic **strains** of ***Saprolegnia.***

Woronina polycystis is an obligate pathogen of *Saprolegnia.* We have isolated strains from ***Saprolegnia*** on adult salmon from three different sites in the Columbia River Basin. As a vigorous pathogen ***in*** the *Saprolegnia* mycelium, it can take over a hemp seed colony of the host in 24 hours. Parasites from all three sites have been isolated into two-membered culture (monoxenic). ***Woronina,*** like most fungi, has two types of reproduction, one fast to exploit the habitat, and the other slow and producing a resistant spore. The resistant spore would be important for biological control. These cystospores are produced in our monoxenic cultures and we are currently attempting to induce their germination. The next logical step would be to attempt to produce them in quantity and test their effectiveness in controlling *Saprolegnia* (zoospore production) on eggs, juveniles and adults.

b. Isolation of bacteria and other microbes that inhibit ***Saprolegnia.***

Hatai (1994) and Olson (Peterson et al., 1994) have both reported on the isolation of bacteria from fish-slime that inhibit growth ***in*** *Saprolegnia.* ***They*** have suggested the possibility of using such strains as microbial control agents of the fungal-fish pathogen. Olson has developed a technique which employs a unique polymer, that gels upon warming, instead of agar which acts in the opposite manner. This permits introduction of test samples into solid media without heating.

Using Dr. Olson's procedures, Dr. Mueller isolated over 30 bacterial cultures that showed definite inhibition of mycelial growth ***in*** *Saprolegnia.* He was not, however, able to confirm the hypothesis that healthy fish had higher numbers of *Saprolegnia* inhibiting bacteria.

2. Development of procedures to monitor *Saprolegnia* treatment programs

a. Inoculum counts.

Dr. Mueller has developed, a direct monitoring procedure for the presence of ***Saprolegnia*** in the water column. Water samples are filtered and saprolegniaceous spores are directly counted with epifluorescence microscopy. This coupled with plating techniques, permitted him to examine the fungal load in Lake Washington and the U.W. Hatchery for one season (Appendix III). The goal here was to have a direct evaluation of spore density in hopes of timing fungicide treatment to the fungal reproductive cycle. This approach could apply to both biological and chemical control approaches.

b. Formalin tolerance.

Some field observations at the Cowlitz Hatchery (Mueller and Whisler, 1994) suggested that the variety of ***strains*** of ***Saprolegnia*** on adult chinook salmon was being reduced during fish-holding and formalin treatment. This could possibly be due to the development of formalin tolerance ***in*** *Saprolegnia.* Although this was not confirmed, procedures for measuring formalin in the water column and fungus response to formalin were developed.

IV. DISCUSSION

The initial experiment demonstrated that following the "ami-momi" treatment, experimental fish developed saprolegniasis. The saprolegniasis that developed was from the strain (292-l) of S. ***parasitica*** added to the tanks. The disease followed the etiology that was expected. The only pathogen observed was S. ***parasitica.*** Thus we believe Koch's postulates were satisfied. The additional experiments described below confirm these fmdings. Thus we conclude that ***Saprolegnia parasitica*** (isolate 292-l) is capable of invading epithelial abrasions in chinook salmon resulting in saprolegniasis that results in the eventual death of the fish. Experiments in Japan using a different species of salmon and a different isolate of ***S. parasitica came*** to the same conclusion (Hatai and Hoshai, 1993, and 1994). In summary every fish that developed lesions, believed to be a result of these experiments, became moribund or died after a mean of 10 days with a mean of body surface involvement of 29%. This suggests that all fish infected with ***Saprolegnia parasitica*** die.

These experiments differ from those in Japan. Using the "ami-momi" treatment and zoospore concentrations of the same order of magnitude, 100% of the fish were infected in the Japanese experiments (Hatai and Hoshai, 1993a, and 1993b). The strain used was isolated from an epizootic of saprolegniasis in Coho salmon. The age of the fish and the population from which the fish were drawn was the similar to those in the epizootic. In our experiments we were unable to achieve more than 60% infection. There are several possible reasons for the difference. We used a strain of ***S. parasitica*** (292-l) that typifies the strains collected from adult chinook salmon after treatment with formalin. The fish from the Cowlitz Hatchery, older and larger in size than the Japanese stocks, were from a stock that could have been exposed to this same pathogen many times. These stocks, in which a few fish develop saprolegniasis, have not been reported to have had an epizootic similar to those reported from Japan. The "ami-momi" treatment may have been quite different and we were unable to confirm the degree of abrasion with the limited number of fish and the time available. The zoospore exposures were also quite different. We exposed the fish

in our experiments for 6 hours and for 24 hours as opposed to the 72 hours used in the Japanese experiments. We believe that these differences can be resolved and that this technique can be used to generate dose/response curves. Such dose/response curves can be used to measure differences in pathogenicity.

The interim infections of ***Saprolegnia*** appear to be the result of aggressive behavior on the part of dominate fish. Low levels of zoospores originating from colonies present in the small amounts of food collected in the bottom of the tanks are suspected to be source of the fungus. Much to our surprise, a few oogonia were observed in the developing fungal mat near the caudal peduncle of the first mortality. We believe that this is the first report of sexual reproduction of ***Saprolegnia parasitica*** outside of laboratory cultivation. Cultures of fungus from this fish and the second fish showed the infecting fungus to be ***S. parasitica*** and RAPD analysis confirms the isolate to be the same as the isolate (292-1) tested in the tank. This isolate (292-1) has been repeatedly tested in the laboratory and has consistently produced oogonia and oospores, but after a few weeks the oospores appear abnormal with eventual break down. ***Saprolegnia parasitica*** was originally described (Coker, 1923) as not having sexual reproduction. Most collections of ***Saprolegnia parasitica*** have come from living fish. Dead fish are not normally collected because saprophytic fungal species quickly invade dead organisms and because the fish quickly disappear after death. The level of individual variability of isolates originating from the Columbia River as determined by molecular techniques suggests that sexual reproduction occurs in ***Saprolegnia parasitica*** in these waters. The discovery of normal oospores in this study supports the contention that sexual reproduction is present in this fungus.

The first social stress experiment demonstrated that the technique has definite potential as a means of testing for pathogenicity. There is no doubt that the fish expected to be submissive, and therefore under stress, were significantly more liable to tail rot and to saprolegniasis than the dominate member of each pair. The condition of the fins was also important in the initial colonization of the fish. ***Saprolegnia*** microcolonies were more common and further developed in the fish with eroded fins than in fish with normal fins (Table 5). The fish from the holding tank that was used as a control had no growth of ***Saprolegnia*** what so ever. The background counts of ***Saprolegnia*** in the water column for a previous year (the only data available) indicates that the "spring bloom" of spores should not have occurred by this time. Examination of the hemp seeds used in this study revealed colonization by *Achyla* sp. and several imperfect fungi but no additional species of ***Saprolegnia*** were recognized. ***Saprolegnia*** *ferax* did appear in the outflow detritus of the "ami-momi" experiments. ***Saprolegnia ferax*** was not recognized in any of the fungal lesion examined. The results of this experiment suggests that ***S. parasitica*** can act as a primary pathogen and infect fish under stress. The fact that two control fish (25%) held individually in baskets also developed and succumbed to saprolegniasis suggests that ***S. parasitica*** can attack normal fish as well.

Fish that were placed at the fourth tank position, where water flow might be considered to be the poorest, showed the greatest mortality from tail rot. The other positions were significantly less affected. It can not be determined if the fourth position was significant for saprolegniasis since eight of the ten experimental fish in that position were gone. The incidence of saprolegniasis appeared to be without positional bias is based on the distribution in the three other baskets. It is possible that water flows through the basket in the fourth position were not as high as the flows through the first three resulting in conditions conducive to tail rot.

The second social stress experiment was established to determine if there was a relationship between tail rot and saprolegniasis. Unfortunately, tail rot did not appear during the second experiment. There are two likely reasons. The temperature remained near 8°C through out the experiment.

The drop in behavior that occurred at the beginning of the first social stress experiment did not occur during the second. A period of cold temperatures may be necessary to initiate this bacterial disease. A second possibility is that the fish were all taken from the same tank. While the numbers of fish present in the holding tanks are reported to prevent the establishment of social order there may have been sufficient recognition factors present such that the level of social stress was lower than that observed in the first social stress experiment. Only some of the changes in behavior that were observed during the first experiment where observed in the second. Changes in parr marks and damage to the fins were present, but the head down or head up and bent body behavior was not seen.

It is possible, that the saprolegniasis occurred as secondary to the bacterial tail rot. Saprolegniasis has been reported as developing secondarily to cold-water disease and other bacterial skin infections (Wolke, 1975; Roberts, 1975). The control fish that developed saprolegniasis did not have the symptoms of the bacterial disease although the disease could have been cryptic.

The fact remains that if the fish had tail rot before contracting saprolegniasis it was not lethal but the ensuing fungal disease did cause extensive tissue destruction which is the most likely cause of death.

Cultures from the fish surface, for the presence of *Saprolegnia*, show a dramatic difference between the dominate fish and the submissive fish. The submissive fish were more extensively colonized. Zoospore cysts easily germinate in 24 four hours on cornmeal agar or in lake water, but the initial hypha is small and is not easy to see, by two days the diameter of the hypha will increase three to four times and begin to branch. This growth pattern suggests that only cysts were present on the dominate fish. Microscopic examination of the mucous of the submissive fish showed the presence of microcolonies and the cultures showed growth within twenty-four hours. This suggests that the turnover rate of the mucous of the submissive fish was slower. The presence of the microcolonies and the differences in the mucous indicate the testing method was functioning.

One of the differences between the first social stress experiment and the second was the presence of uncolonized hemp seeds. Hemp seeds are used because they act as excellent substrates for *Saprolegnia*. Hemp seeds are one of the few natural substrates on which sexual reproduction is known to occur. These hemp seeds were immediately colonized by *Saprolegnia ferax* and other molds. The experiment was started about the time the spring bloom of *Saprolegnia* zoospores appears in the water column The non-treatment experimental fish were then exposed to very high levels of *S. ferax*. *Saprolegnia ferax* has been reported many times as a parasite of non-salmonid fish. It was found twice in the first phase of this project (Mueller and Whisler, 1994). It is not surprising under the conditions present that S. *ferax* was able to infect fish in this experiment. The reasons for the success of the first social stress experiment and the partial failure of the second are not understood at this time.

The "ami-momi" and the social stress experiments appear to be excellent choices for testing the patbogenicity of *Saprolegnia* or other diseases. These two methods more accurately reflect the conditions fish actually experience. Injection and loading the food with the infective agent are useful methods, but lack the potential sensitivity necessary to understand the infective process.

The results of these two sets of experiments show that (1) saprolegniasis is a lethal disease of salmon and (2) the experimental methods have the potential to test for differences in strain pathogenicity .

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RAPD PROCEDURE

1. PCR the samples.
2. If you are using a single primer then make the Single Primer PCR Cocktail found at 4a. If you are using two primers then make the Double Primer PCR Cocktail found at 4b.
   (ul means microliters)
3. Make the PCR Cocktail

   If you want to PCR more than one sample then multiply all the amounts of the reagents by the number of samples plus two, one for the negative control and one to make sure that you do not run out of PCR cocktail.

4a. Single Primer PCR Cocktail

Sterile H2O	28.5 ul
Primer	4.0 ul
50% glycerol	5.0 ul
dNTP	5.0 ul
Taq	0.5 ul

4b. Double Primer PCR Cocktail

Sterile H2O	28.5 ul
Primer #1	2.0 lll
Primer #2	2.0 ul
50% glycerol	5.0 ul
dNTP	5.0 ul
Taq	0.5 ul

5. Vortex the PCR cocktail for three seconds.
6. If you made the cocktail for more than one sample divide the cocktail into 48 ul aliquots.
7. Add 2 ul of sample DNA into one of the 48 ul cocktail aliquots, one 48 ul cocktail aliquot for one DNA sample. There should be two tubes that no DNA was added to, one is the negative control. The other you can discard or use if you spilled a tube.
8. Add one drop of mineral oil to each tube.
9. Put samples into PCR machine

RAPD PROCEDURE

1. PCR the samples.
2. If you are using a single primer then make the Single Primer PCR Cocktail found at 4a. If you are using two primers then make the Double Primer PCR Cocktail found at 4b.
(ul means microliters)
3. Make the PCR Cocktail

If you want to PCR more than one sample then multiply all the amounts of the reagents by the number of samples plus two, one for the negative control and one to make sure that you do not run out of PCR cocktail.

4a. Single Primer PCR Cocktail

Sterile H20	28.5 ul
Primer	4.0 ul
50% glycerol	5.0 ul
dNTP	5.0 ul
Taq	0.5 ul

4b. Double Primer PCR Cocktail

Sterile H2O	28.5 ul
Primer #1	2.0 ul
Primer #2	2.0 ul
50% glycerol	5.0 ul
dNTP	5.0 ul
Taq	0.5 ul

5. Vortex the PCR cocktail for three seconds.
6. If you made the cocktail for more than one sample divide the cocktail into 48 ul aliquots.
7. Add 2 ul of sample DNA into one of the 48 ul cocktail aliquots, one 48 ul cocktail aliquot for one DNA sample. There should be two tubes that no DNA was added to, one is the negative control. The other you can discard or use if you spilled a tube.
8. Add one drop of mineral oil to each tube.
9. Put samples into PCR machine

10. Run them on profile Wl : (The temperature is in Celsius)

94 5 minutes }
36 5 minutes } X2
72 5 minutes }

94 1 minute }
36 2 minutes } *X40*
72 **2 minutes}**

72 10 minutes
4 until you turn off the machine

11. Take the samples out of the machine and store them in the freezer until it is time to load them.
12. Make **a 2%/1%** gel
13. Pour 60 ml of 1X TAE into a clean flask.
14 Add 1.2 grams Nusieve.
15. Add 0.6 grams **SeaKem.**
16. Seal the top of the flask with aluminum foil.
17. Put the flask into a hot pot of hot water. Let it stay in there for twenty minutes until the mixture is homogeneous, clear with no mixing lines.
18. Pour the hot gel into a level gel tray with its combs in place.
19. Allow the gel to solidify for twenty minutes.
20. Place gel in the **refrigerator** for five minutes.
21. Carefully pull out the combs. Try to avoid ripping out the bottom of the gel.
22. Put gel with gel tray in the electrophoresis machine with 730 ml 1X TAB **buffer.**
23. Add 7 **ul** of 1 KB ladder to the second well. Try to avoid adding samples to the edge wells of gels because the samples tend to run at a different rate at the edges of gels.
24 Mix 7 **ul** of sample with **.75** ul-1 .O **ul** loading dye and load the mixed samples into the wells. Keep track of which samples went into which wells.
25. Once all the samples have been loaded add another 7 **ul** of 1 KB to the empty well adjacent to the last well loaded with sample.
24. **Run** the samples through the gel at 94 volts for one and a half hours.
25. Wash the gel with 7 **ul** of Ethidium Bromide and 100 **ml** of water for 20 minutes.
26. Pour the Ethidium Bromide contaminated water into the Ethidium Bromide liquid waste container.
27. Wash the gel in 100 ml of water for 20 minutes.
28.' Take the gel **downstairs** to the *W.* transilluminator and take a picture of it.

18s rRNA SEQUENCE OF ISOLATE 291-1 COMPARED TO *ACHLYA*

KEY: ACKAC=*Achlya ambisexualis;* Other rows Isolate 291-1

APPENDIX IIa

```
              5          15         25         35         45
HCWJ19    G--------- ---------- ---------- ---------- ----------
ACKAC     ACCTGGTTGA TCCTGCCAGT AGTCATACGC TTGTCTCAAA GATTAAGCCA
HCWMr22,  CCC------- -CCCCCC--- --CCACCCCC CCCCCCC--- --------CC-
HCWMy26,  ---------- -----CCA-- ---CA-ACG- -------AGG AATT------

              55         65         75         85         95
HCWJ19    m---m----- ---------- ---------- ---------- ----------
ACKAC     TGCATGTCTA AGTATAAACA ATTTTGTACT GTGAAACTGC GAATGGCTCA
HCWMr22,  ------CCC- ---------- ---------CC CCAAAACCCC CACCAACTCA
HCWMy26,  ------CCTA G---TAAACG A--------- ---------- -AACG-----

              105        115        125        135        145
HCWJ19    ---------- ---------- ---------- ---------- ----------
ACKAC     TTATATCAGT TATAGTCTAC TTGATAGTAC CTTACTACTT GGATAACCGT
HCWMr22,  TT-------- ------CCAA TTGCCAG--- ------ACTT GAA-------
HCWMy26,  ---------- ---------- ---------- ---------- ----------

              155        165        175        185        195
HCWJ19    ---------- ---------- ---------- ---------- ----------
ACKAC     AGTAATTCTA GAGCTAATAC ATGCGTAAAT ACCCAACTGC TTGTCGGACG
HCWMr22,  ---AATCCC- ---------T ACGCAT---- -TCCAATTGC ----CAGAC-
HCWMy26,  AGGAATTC-- ---CTAGTA- ---------- ---------- ----------

              205        215        225        235        245
HCWJ19    ---------- ---------- ---------- ---------- ----------
ACKAC     GGTAGCATTT ATTAGATTGA AACCAATGCG GCTTCGGTCG GTATTGTGTT
HCWMr22,  ---------- ------TTGA AA-------A GCC-----CG GCATTGT---
HCWMy26,  ---------- ---------- ----AACGCA ---------- ----------

              255        265        275        285        295
HCWJ19    ---------- ---------- ---------- ---------- ----------
ACKAC     GAGTCATAAT AACTGTGCGG ATCGCTTTTA GCGATACATC AATTGAGTTT
HCWMr22.  ------TA-- ---------- ------TTTA TTG------T CACTACCTCC
HCWMy26,  -AGTCAT--C AGCT------ ---------- ----TGCATT GATTACGTCC

              305        315        325        335        345
HCWJ19    ---------- ---------- ---------- ---------- ------TAAC
ACKAC     CTGCCCTATC AGCTTTGGAT GGTAGGATAT GGGCCTACCA TGGCGTTAAC
HCWMr22.  CTG---TGTC AGGATTGGGT AATT---TAC GCGCCTGCT- --GCCTT---
HCWMy26,  CTGCCCT--- ----TTG--- ---------- -----TAC-- --------AC

              355        365        375        385        395
HCWJ19    GGGTAACGGG GAATTAGGGT TCGATTCCGG AGAGGGAGCC TGAGAAACGG
ACKAC     GGGTAACGGG GAATTAGGGT TTGATTCCGG AGAGGGAGCC TTAGAAACGG
HCWMr22,  ---------- ---------- ---------- --------CC TTGGATGTGG
HCWMy26,  -----AC--- ---------- ---------- ---------- -------CGC

              405        415        425        435        445
HCWJ19    CTACCACATC CAAGGAAGGC AGCAGGCGCG CAAATTACCC AATCCCGACA
ACKAC     CTACCACATC CAAGGAAGGC AGCAGGCGCG TAAATTACCC AATCCTGACA
HCWMr22,  TAGCCGTTTC TAAGG----- ---------- ----CT-CCC TCTCCGGA--
HCWMy26,  CCGTCGCACC TA-------- ---------- --------CC GAT-------
```

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              455        465        475        485        495
HCWJ19    CAGGGAGGTA GTGACAATAC ATAACGATAC ---------- ----------
ACKAC     CAGGGAGGTA GTGACAATAA ATAACAATGT CGGGCTTTTT AAGTCTGACA
HCWMr22,  ---------- ---------- ---------AT CAAACCCT-- AATTTC--GC
HCWMy26,  ---------- -------TGA ATGAC----T CGG-----TG AAGT------

              505        515        525        535        545
HCWJ19    ------ACGG G--------- ---CCCTT-- ---------- TT------CA
ACKAC     ATTGGAATGA GAACAATTTA AATCCCTTAA CGAGGATCAA TTGGAGGGCA
HCWMr22,  GTT------- ---------- -ACCCGTTAA CG----CCA- -TGGAGG---
HCWMy26,  ATTGGGACTG TG--AATTTG TGTGCTTCA- ---------- TTGCATG-CA

              555        565        575        585        595
HCWJ19    GGTCT--TGC TA-------- ---------- ---------- ----------
ACKAC     AGTCTGGTGC CAGCAGCCGC GGTAATTCCA GCTCCAATAG CGTATATTAA
HCWMr22,  ---------- ---------- ------CCCA TATCC--TAC CAT---CCAA
HCWMy26,  AGTTTG---- TGGGAAC-GA GG-AATTCC- -------TAG TAAACGC--A

              605        615        625        635        645
HCWJ19    --TTGTTACG G--------C CGG------- ---------- ----------
ACKAC     AGTTGTTGCA GTTAAAAAGC TCGTAGTTGG ATTTCTGGTT TGAGCGTCCG
HCWMr22,  AGCTGATAGG GC--AGAAAC TC--AATTG- ACTT------ ----------
HCWMy26,  AGTCATC--- -------AGC TTGCA----- ----TTGATT ---ACGTCC-

              655        665        675        685        695
HCWJ19    ---------- ---------- ---------- ---------- ----------
ACKAC     GTCGAGTTTA TCTCTGTACT ATGGATGTTT GGGCCATTTT TTGTGAGGAT
HCWMr22,  ATCG------ ---CTGTGTG ATCAGTATTT -------CTG TCAC-----T
HCWMy26,  ------CTGC CCTTTGTAC- ----ACA--- ---CCGCCCG TCGCA-----

              705        715        725        735        745
HCWJ19    ---------- ---------- ---------- ---------- ----------
ACKAC     GCTTTTCTGC CATTCAGTTG GTTGGTGAGT AGACTTGCAT CGTTTACTGT
HCWMr22,  ACCTCCCCGT ------GTCG --GATTGGGT A-ATTTGCG- CGCCTGCTGC
HCWMy26,  -----CCTAC C--------- --GATTGAAT -GACTCG--- --------GT

              755        765        775        785        795
HCWJ19    --AAAAGTT- ---------- --CAGACGTT AACTTACTC- ----------
ACKAC     GAAAAAATTA GAGTGTTTAA AGCAGGCGTT TGCTCATTTG AATACATTAG
HCWMr22,  ---------- ---------- ---------- --CTTCCTTG GATG------
HCWMy26,  GAAGTATTGG GACTG--TGA A-------TT TGTGTGCTT- ----CATTT-G

              805        815        825        835        845
HCWJ19    ---------- ---------- ---------- -----TTCTT ----------
ACKAC     CATGGAATAA TAAGATACGA CCTTGGTGGT CTATTTTTGTT GGTTTGCACA
HCWMr22,  ---------- ---------- ----TGGTAGC C--------- -GTTT-----
HCWMy26,  CATG------ CAAG------ ---------- ----TTTTGTG GG--------

              855        865        875        885        895
HCWJ19    ---------- ---------- ---------- ---------- ----------
ACKAC     CCGAGGTAAT GATTAATAGG GACAGTTGGG GGTATTCATA TTTCAACGTC
HCWMr22,  ---------- ---------- ---------- ----CTCAGG CTCCCTCTCC
HCWMy26,  ---------- ----AACTGG ----GCCGGG AACTTTC--- -CTTAACCTC
```

```
          905         915         925         935         945
HCWJ19    -------AAA  TT--------  ----------  ----------  ----------
ACKAC     AGAGGTGAAA  TTCTTGGATC  GTTGAAAGAT  GAGCTTAGGC  GAAAGCATTT
HCWMr22,  GGAG------  ----------  -TCGAAGGGT  AA--TTGGG-  ----------
HCWMy26,  G---------  ---------C  ATTTAGAGGAG AAGT-----C  GAA-------

          955         965         975         985         995
HCWJ19    ----------  ----------  ----------  ----TAGGGA  ATTG------
ACKAC     ACCAAGGATG  TTTTTCATTAA TCAAGAACGA A  AGTTAGGGG  ATCGAAGATG
HCWMr22,  ----------  ----------  ----------  GTTTAGGG-  -TTGAAAATG
HCWMy26,  --CAAGG---  --TCCGT---  ----------  ----------  -----AGGTG

          1005        1015        1025        1035        1045
HCWJ19    ----------  ----------  ----------  ---------C  TC--------
ACKAC     ATTAGATACC  ATCGTAGTCT  TAACCATAAA  CTATGCCGAC  TCGGGATTGG
HCWMr22,  ATT-------  -------TTT  TAATT-----  ---------C  CTTGGATGTG
HCWMy26,  A------ACC  AT-ATAACCT  TGATCG----  --ATA--GGT  CTGG-----G

          1055        1065        1075        1085        1095
HCWJ19    ----------  ----------  ----------  ----------  ----------
ACKAC     CAGTCGTTTT  TTTTGAATGAC CT-l-GTCAGCA CCGTATGAGA  AATCAAAGTC
HCWMr22,  TAGCCGTTTC  T----AAGGC  -?-ccCTC---  -----TCCGG  AATCAAACCC
HCWMy26,  TAATC----T  TTTTGAAT-AC -------GTA  TCGTGCTAGG  GAT-------

          1105        1115        1125        1135        1145
HCWJ19    ----------  ----------  ----------  ----------  ----------
ACKAC     TTTGGGTTCC  GGGGGGAGTA  TGGTCGCAAG  GCTGAAACTT  AAAGGAATTG
HCWMr22.  T---AATTCC  ----------  CCGTTAC---  -CCG------  -------TTA
HCWMy26,  ---AGATT--  ----------  --ATTGCAA-  --------TT  A---------

          1155        1165        1175        1185        1195
HCWJ19    ----------  ----------  ----------  ----------  ----------
ACKAC     ACGGAAGGGC  ACCACCAGGA  GTGGAGCCTG  CGGCTTAATT  TGACTCAACA
HCWMr22,  ACG-------  -CCAT----G  GTAG-GCCCA  TATCCTA---  ----------
HCWMy26,  ----------  ----------  ----------  ----TTAAT-  ---CTTGA-A

          1205        1215        1225        1235        1245
HCWJ19    ----------  ----------  ----------  ----------  ----------
ACKAC     CGGGGAAACT  TACCAGGTCC  AGACATAGTA  AGGATTGACA  GATTGAGAGC
HCWMr22,  ----------  --CCA--TCC  AAAGCTGATA  GGG-----CA  G-----AAAC
HCWMy26,  CGAGGAA--T  TCCTAG---T  AAAC---GCA  AG--TCATCA  GCTTG-----

          1255        1265        1275        1285        1295
HCWJ19    ----------  ----------  ----------  ----------  ----------
ACKAC     TCTTTCTTGA  TTCTATGGGT  GG TGGTGCAT GGCCGTTCTT  AGTTGGTGGA
HCWMr22,  TC--------  ----------  ----------  ----------  ---------AA
HCWMy26,  --- -CATTGA             AGT  CCCTGCCCTT  TGT-------

          1305        1315        1325        1335        1345
HCWJ19    ---------C  TAGTTAACTC  C---------  ----------  -CGT------
ACKAC     GTGATTTGTC  TGGTTAATTC  CGTTAACGAA  CGAGACCTCC  GCGTGCTAAA
HCWMr22,  TTGACTTATC  ----------  -GCTG-TGAA  ---GACC---  GCAC----AG
HCWMy26,  ---AC-----  ------ACAC  CGC-------  -----CCGTC  GCAC------
```

```
              1355        1365        1375        1385        1395
HCWJ19     ----------  ----------  ----------  ----------  -------TCA
ACKAC      TAGTTCTGCT  TACCAATTTG  GTAGGTAACG  ACTTCTTAGA  GGGACTTTCA
HCWMr22,   TTATTATGAT  T--CAACACA  ATACCTGAG-  -TTTCTCAGG  ATCCCTCTCC
HCWMy26,   ----------  CACCGATTT.  ----------  ----------  ----------

              1405        1415        1425        1435        1445
HCWJ19     G---------  ----------  ----------  ACACGTC---  ----------
ACKAC      GTGACTAACT  GAAGGAAGTT  GGAGGCAATA  ACAGGTCTGT  GATGCCCTTA
HCWMr22,   G---------  -----GAGTC  GAA-------  ------CCCT  AATTCCCCT-
HCWMy26,   --GAATGACC  G---------  ----------  ----------  ----------

              1455        1465        1475        1485        1495
HCWJ19     ----------  GTCG---GCG  CATTA-----  ----------  ----------
ACKAC      GATGTTCTGG  GCCGCACGCG  CGCTACACTG  ATACGCTCAA  CGAGTATATA
HCWMr22,   ----------  ----TACCCG  ----------  ------TCAA  -AAGCATGTG
HCWMy26,   ----------  --CGCACGCG  -GCTACACTG  ATACGCTCAA  CGAGTATATA

              1505        1515        1525        1535        1545
HCWJ19     ----------  --AGGTCGAG  GT--------  -------TAT  CGC-------
ACKAC      ACCTTGATCG  ATAGGTCTGG  GTAATCTTTT  GAATACGTAT  CGTGCTAGGG
HCWMr22,   A---------  --------GG  GTGACCCT--  -----CACAT  C---------
HCWMy26,   ACCTTGATCG  ATAGGTCTGG  GTAATCTTTT  GAATACGTAT  CGTGCTAGGG

              1555        1565        1575        1585        1595
HCWJ19     ATA-------  --------TA  AT--------  ----------  ----------
ACKAC      ATAGATTATT  GCAATTATTA  ATCTTGAACG  AGGAATTCCT  AGTAAACGCA
HCWMr22,   ----------  ---------A  AACTTGATAG  GGCAGTCCCT  CTC---CG-G
HCWMy26,   ATAGATTATT  GCAATTATT-A ATCTTGAACG  AGGAATTCC-  AGTAAACGCA

              1605        1615        1625        1635        1645
HCWJ19     ----------  ----------  ----------  ----------  ----------
ACKAC      AGTCATCAGC  TTGCATTGAT  TACGTCCCTG  CCCTTTGTAC  ACACCG
HCWMr22,   AGTCG--AAC  CCTCATT---  ----------  CCCCTTA---  ------
HCWMy26,   AGTCATCAGC  TTGCATTGAT  TACGTCCCTG  CCCTTTGTA-  ----------

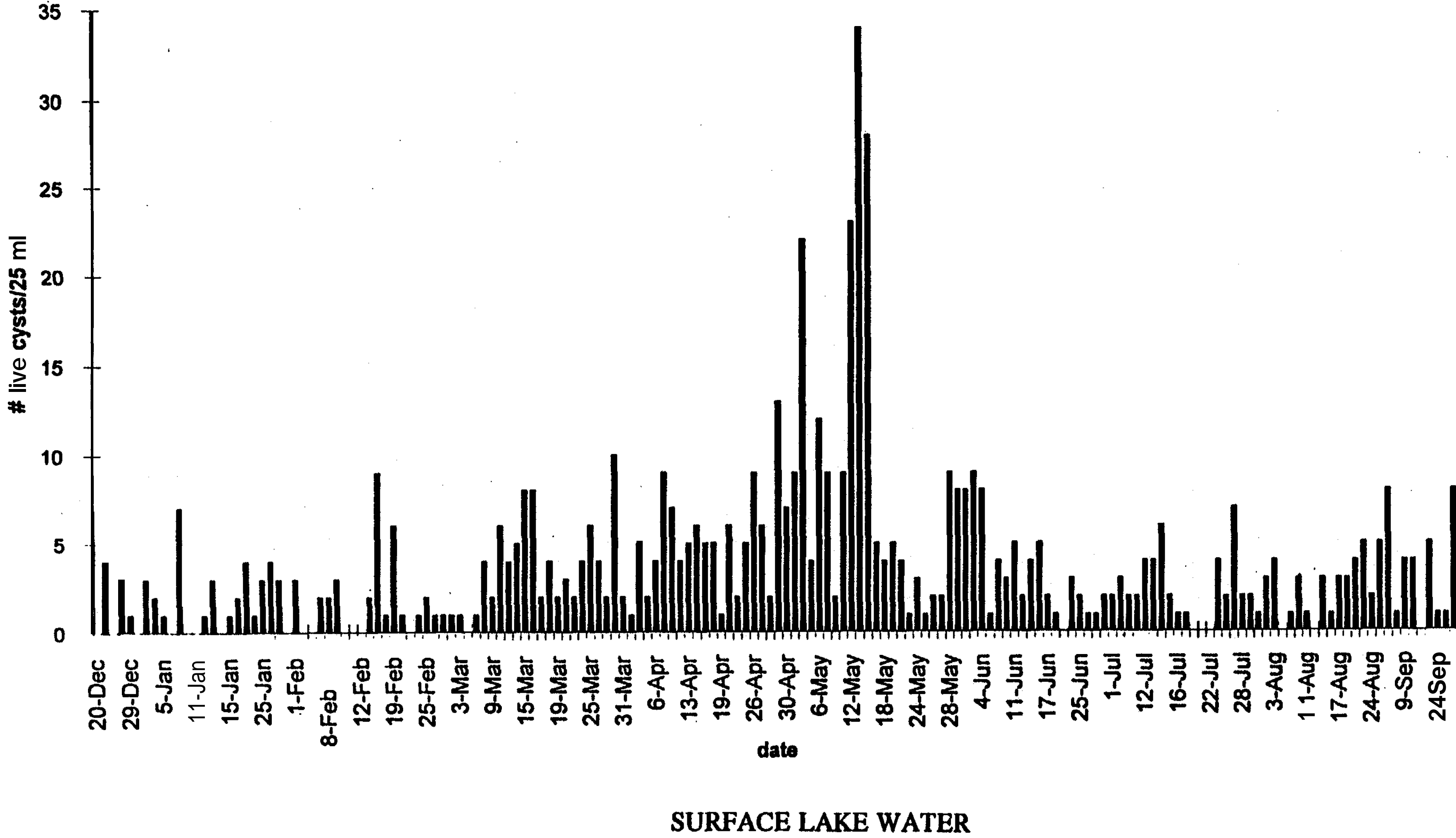
              1655        1665        1675        1685        1695
HCWJ19     ----------  ----------  ----------  ----------  ------TTCT
ACKAC      TCGCACCTAC  CGATTGAATG  ACTCGGTGAA  AAATTGGGAC  CGTTAATTCT
HCWMr22,   TC--------  ------AAAA  GCATG....   ...*TGAGGG  CGTAGG----
HCWMy26,   ----------  ----------  ----------  ----------  ----------

              1705        1715        1725        1735        1745
HCWJ19     CTC-------  ----------  ----------  ----------  ----------
ACKAC      CTTGCTTCAT  TGTGAGTCAG  TTGATGGGAA  CTTTTTTTAA  CCTCGCCA TT
HCWMr22,   ----------  .GTGAG----  ---ATGGAAA  CTT-------  ----------
HCWMy26,   ----------  ----------  ----------  ----------  . ..-.*....

              1755        1765        1775        1785        1795
HCWJ19     ----------  ----------  ----------  ----------  ----------
ACKAC      TAGAGGAAGG  TGA AGTCGTA ACAAGGTTTC  CGTAGGTGAA  GCTGCGGAAG
HCWMr22,   --------GA  TAGGGC---   ---AGGTATT  TG--AATGAT  C---------
HCWMy26,   ----------  ----------  ----------  . ...**.*.*  ----------
```

COUNT OF SAPAROLEGNIACEOUS CYSTS IN SURFACE WATER LAKE WASHINGTON, WA

NOTE: When tested, these cysts were not *S. parasitica*, the fish parasite, but other species of the Saprolegniales.



SURFACE LAKE WATER