

Characterization of *Saprolegnia* spp. isolates from infected eggs, fry and adults of common carp *Cyprinus carpio* L. based on molecular data in Al-Manahel and Al-Wahda fish hatcheries, in middle of Iraq
توصيف انواع مختلفة من الفطر *Saprolegnia* من بيوض وافراخ وبالغات اسماك الكارب الاعتيادي *Cyprinus carpio* L. بالاعتماد على البيانات الجزيئية في مفاقي اسماك المناهل والوحدة وسط العراق

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Abstract

The aim of this study was to identify *Saprolegnia* spp. isolated from infected common carp *Cyprinus carpio* L. eggs and fry in two fish hatcheries (Al-Manahel and Al-Wahda) in the middle part of Iraq, during the period from March-June 2009. It was evident from molecular diagnosis (PCR Technique) that isolates of the genus *Saprolegnia*, shared one feature (production of secondary zoospores with long hooked hairs). These isolates were divided into four groups based on the findings of molecular diagnostics PCR. Isolates of *Saprolegnia* spp. were characterized genetically and physiologically. The majority (25 from 45) of the isolates in both hatcheries were almost genetically identical as assessed by RAPD-PCR. The remaining isolates belonged to three different groups.

Key words: molecular data, fish hatcheries, *Saprolegnia* spp

المستخلص

الهدف من هذه الدراسة هو تشخيص انواع مختلفة من الفطر *Saprolegnia* المعزولة من اسماك الكارب المصابة في مفاقي المناهل و الوحدة خلال الفترة من شهر اذار ولغاية حزيران 2009. تم مقارنة 45 عزلة من الجنس *Saprolegnia* و التي تمتاز انتاجها السبوروات السابحة ذات الاشواك ، قسمت الى اربعة مجاميع بالاعتماد على نتائج التشخيص الجزيئي باستخدام جهاز الـ (PCR) تتكون المجموعة الاولى من 25 عزلة من اصل 45 عزلة، تحتوي المجموعة على العزلات المم رضة المعزولة من الاسماك المصابة بالفطر *Saprolegnia* وبذلك يمكن استخدام تقنية الـ (PCR) لدراسة الترابط الوراثي لعزلات الفطر المختلفة وفهم تواجدتها وانتشارها .

الكلمات المفتاحية : البيانات الجزيئية ، مفاقي اسماك ، *Saprolegnia* spp

Introduction

Today, molecular methods, especially polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism analysis (RFLP) is one of the most important methods to identify different *Saprolegnia* species and it could distinguish *S. parasitica* from *S. diclina*. In addition, RAPD-PCR had applied for analysis of the fish pathogenic *Saprolegnia* genome. In the last few years, molecular tools have been developed that can be applied also to Oomycetes. Using random amplified polymorphic DNA-PCR (RAPD-PCR) which has been described by [1] in this technique, single or a pair wise combination of primers, typically 9-10 nucleotides in length, are used to amplify target genomic DNA by polymerase chain reaction (PCR). Fragments of DNA are generated by PCR amplification if the primer target sites for the primer happen to occur within approximately 5 kb of each other on opposite DNA strands. Amplified products, which form strain-specific fingerprints, are then analyzed by separation through an agarose gel and ethidium bromide stained. Randomly amplified polymorphic DNA (RAPD) analysis seemed to be efficient in distinguishing different isolates; it has a high discriminatory power, it is easy to perform, does not require radiolabel led probes, and it is applicable to several microorganisms [2]. In the fermentation industry, this procedure has been useful for the characterization of different isolates of *Fusarium* [3].

There are other techniques to study the genetic diversity of Oomycete pathogens and thus reveal population structures [4,5]. Techniques such as differential screening or differential display Reverse

البحث مستل من اطروحة الدكتوراه للباحث الثالث

Transcriptase-PCR [6] can be used to find genes expressed at certain time points also in organisms where genetic approaches or screening for mutants are not possible. [7] used internal transcribed spacer (ITS) region and the 5.8S rRNA, they found a group of currently identified *Saprolegnia* species including *S. litoralis* and *S. ferax* shared an almost identical ITS sequence (above 99% similarity) with that of isolate. Amplification of DNA by (PCR) using oligonucleotides as primers appears to be useful for taxonomic studies at levels ranging from populations to species [1,8]. Random amplification polymorphic DNA (RAPD)-PCR has been used within the Oomycetes to distinguish both different strains and species. For example, in a closely related species *Aphanomyces astaci*, a crayfish pathogen, genetic diversity among several isolates were studied by this technique [9]. Recently, it was showed that a strain of *Aphanomyces astaci* exhibited physiological properties different from those shown by previously characterized strains of *Aphanomyces astaci* and the application of RAPD-PCR demonstrated that this isolate were genetically separated from all other hitherto described strains [10]. RAPD-PCR has also been used in some other species of Oomycetes, i.e. *Phytophthora* spp. [11] and *Saprolegnia* by [12]. The aim of this study usage of RAPD-PCR technology to study phylogenic relationship of 45 *Saprolegnia* isolates from different habitat and to facilitate the understanding of their predominance.

Materials and Methods

Isolation of *Saprolegnia* from Eggs, fry and adults

In this study, *Saprolegnia* spp. isolates tested by RAPD-PCR were isolated from infected carp fish (adults, fry and eggs) and from water, isolation of *Saprolegnia* collected from both fish and water were carried out between March-July 2009 during the artificial propagation in two fish hatcheries (Al-Manahel and Al-Wahda) in the middle part of Iraq, as the method stated by [13]. During incubation period eggs collected from incubators randomly, eggs coated with fungal mycelium collected in 100ml sterile glass bottles filled with sterile distilled water and transferred to the laboratory. On sampling time, water temperature was 17-19 °C. Isolation of *Saprolegnia* from adults was carried out from naturally infected fish. Samples were collected from fish showing skin lesions, eye, fins, gills, mouth and inoculated onto Corn Meal Agar (CMA) medium plates and incubated at 20 ± 2°C for 3-4 days, subculture on the same media was done for purification.

Isolation of *Saprolegnia* from water

Baiting method which adapted by [14] was used to collect *Saprolegnia* spp. using sesame seeds *Sesamum indicum*.

Pure Culture Preparation for Morphological Diagnosis

The samples were washed several times in sterile distilled water, and egg membranes with attached fungal hyphae were separated and washed again in sterile distilled water three times. Then, the egg membranes were placed in glass Petri dishes (5-10 eggs per plate) containing 25-40 ml of sterilized distilled water with Chloramphenicol and Gentamycin at a concentration of 100µg/ml to prevent bacterial contamination and maintained in hatcheries at 18 °C for one day. Then, the egg membranes directly were inoculated in CMA agar plates and incubated in the same temperature for 3-5 days.

Preparing *Saprolegnia* Isolates for RAPD-PCR

These forty five *Saprolegnia* isolates were selected that having secondary cysts with long hairs, depending on previous studies considered *Saprolegnia* isolates secondary cysts with long hairs as potential pathogenic for fish [15].

Saprolegnia isolates were used in RAPD-PCR Table (1) are:

- a:** Two isolates with antheridia and investigated oogonia (*Saprolegnia* IO) and indirect germination obtained from cutaneous lesions of carp fish adults formerly identified as *S. parasitica*.
- b:** Twelve isolates non-investigated oogonia (*Saprolegnia* O) with little or no indirect germination and scarce secondary cysts. From carp lesions and water.
- c:** Thirty one asexual *Saprolegnia* with abundant indirect germination from water and infected eggs and larvae.

Table (1): Characteristics and origin of the 45 long-hairs *Saprolegnia* isolates.

Number of isolates	Host: habitat			Isolate
	Water	Mucus	Cutaneous lesion	
2			Carp adults	<i>Saprolegnia</i> IO ⁽¹⁾⁽²⁾ (with invested Oogonia)
2		Carp adults		
1			Fins	
1			operculum	<i>Saprolegnia</i> O ⁽³⁾⁽⁴⁾ (with non Invested Oogonia)
8	Water			
13			Fry	
12		eggs		
6	water			
45	14	16	15	Total

⁽¹⁾ Formerly identified as *S. parasitica*.

⁽²⁾ Abundant indirect germination.

⁽³⁾ Non antheridial invested oogonia. Isolates quite similar to the former *S. diclina*.

⁽⁴⁾ Little or no indirect germination and scarce secondary cysts.

Preparation of genomic DNA from *Saprolegnia* Mycelium

DNA isolation

A small piece of mycelium (approximately 1.5 mg dry weight) grown in PG-agar culture for three days, the mycelia were harvested, washed with sterile distilled water and ground in liquid nitrogen with a mortar. Total genomic DNA was extracted as described by [16]. DNA extracts were stored at -20 C⁰. The DNA was confirmed by running each sample on 1% agarose gel with Ethidium bromide 0.5 µg/mL for 1 hour 70 Volts/ cm².

RAPD-PCR

PCR was carried out in 25 µl volumes of 1X PCR buffer contains 100 ng mycelia DNA approximately 1.2 µl, 12.5 µl master mix 10 mM Tris-HCl (pH 8.60), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM of each dNTP and 2.5 units of Taq DNA polymerase) (Sinagen Company), 0.4 mM prime approximately 1.3 µl (Sinagen Company) and 10 µl distilled water. Amplification was performed in thermo cycler PCR (Thermo) programmed for one cycle of initial denaturation at 95 C⁰ for 5 min, 40 cycle of denaturation 94 C⁰, annealing at 38 C⁰ for 45 s and extension at 72 C⁰ for 1.5 min and final extension at 72 C⁰ for 10 min. Ten ml of PCR product were separated on 1.5% agarose gel and 100 bp ladder DNA (Fermentans) was used as the molecular weightmarker. Gels were stained with ethidium bromide, visualized UV illumination at 336 nm, and photographed with a Polaroid camera. PCR mixture without DNA template was used as negative control. Table (2) showed the following primers were used.

Table (2): Primers used in the PCR Technique.

Primer signal	Sequence and direction	Reference
A06	5'-GGTCCCTGAC-3'	[17]
A07	5'-GAAACGGGTG-3'	[17]

Data Treatment and Statistical Analysis.

Similarity coefficients (F) between 2 isolates were calculated according to the formula of [18] $F = \frac{2N_{xy}}{N_x + N_y}$, where N_{xy} , is the number of common fragments between 2 isolates, and N_x and N_y , are the number of fragments in isolates X and Y, respectively. Pooled data from 8 primers were used for this calculation.

Results

Amplification of *Saprolegnia* isolates genomic DNA in preliminary experiments, the 2 primers listed in 'Materials and methods' gave consistent results and produced a reasonable number of identifiable and polymorphic bands. They produced in total 722 clearly amplified PCR bands in 62 different positions, and the average number of bands per isolate was 33.5. The number of amplified DNA fragments generated by each RAPD primer ranged from 2 when primer A06 was used to 10 in case of using primers A07. The size of the fragments produced ranged from 180 to 1500 bp and there were 38 positions, i.e. 31% of total, with a product size larger than 1 kb. An example of an RAPD pattern generated by primer A07 showed in Figure (1-A).

The RAPD profiles obtained by using the A06 primer on 45 *Saprolegnia* spp. isolates gave two distinguishable PCR markers Figure(1-B). Of these, two fragments, i.e. 380 and 1100 bp, were consistently present in all isolates. It is possible that the 380 bp product could be useful for the development of diagnostic tools within the *S. parasitica-diclina* complex.

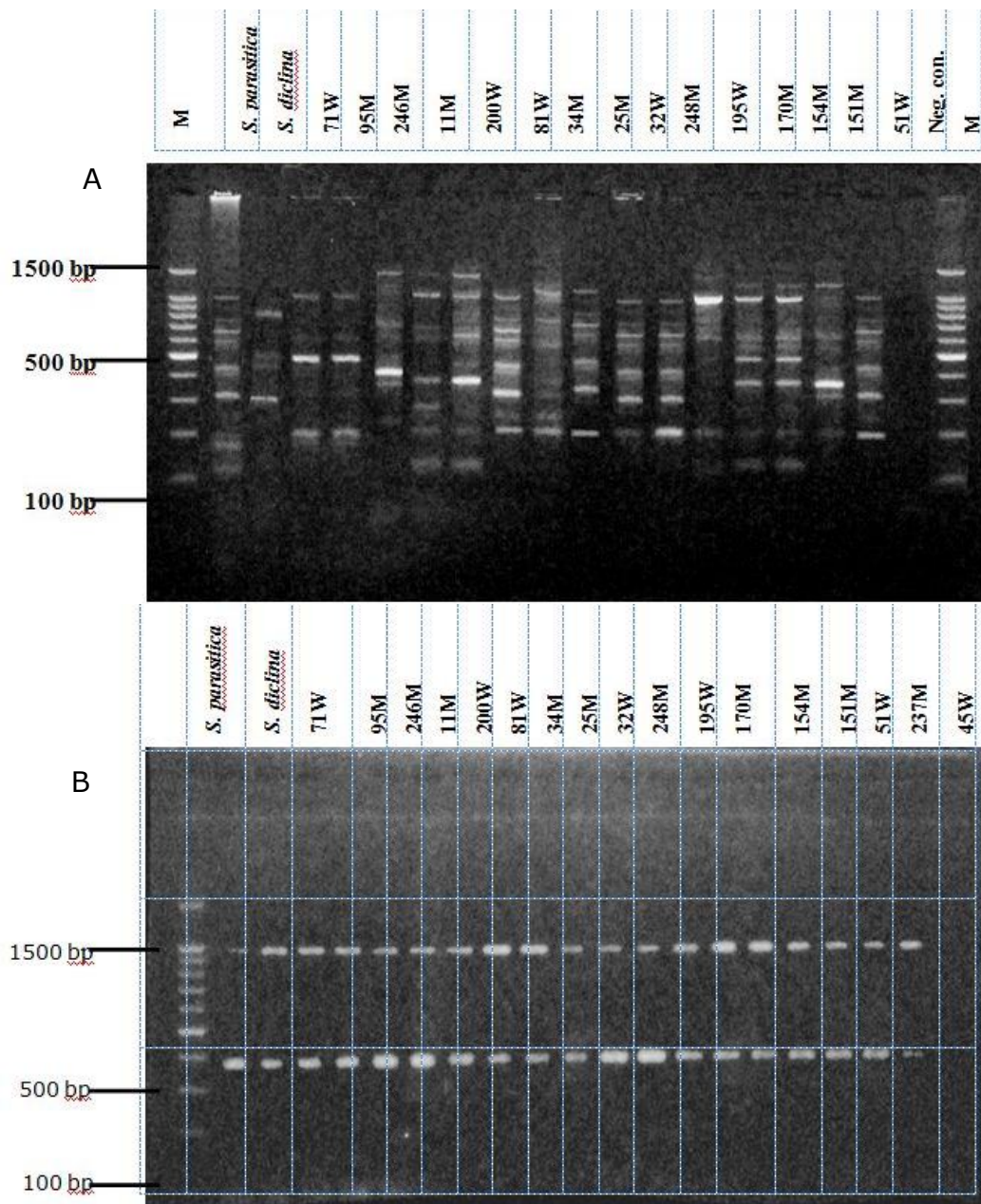


Fig. (1): Amplification of genomic DNA from different *Saprolegnia* spp. isolates. (A): using the primer A07. (B): using the primer A06. 100 bp ladder size marker (M), *S. parasitica*, *S. diclina*, 71W, 95M, 246M, 11M, 200W, 81W, 34M, 25M, 32W, 248M, 195W, 170M, 154M, 151M, 51W, 237M, 45W.

Genetic Variation among Isolates Based on Analysis of RAPD Pattern

All PCR fragments obtained were utilized for genetic distance analysis. A similarity matrix indicated that the 45 *Saprolegnia* isolates which share one feature (production of secondary zoospores with long hooked hairs), can be grouped into four distinct groups Table (3).

The first group is the largest group contain 25 from 45 isolates (32M through 172W, 245M, 247M, 248M, 22W, 1W, 2W, 3M, 4M, 6M, 7M, 8M, 9M, 10M, 12W, 13W, 15M, 17M, 25W, 48W, 70M, 154M, 195W, 210W).

The second group is formed by 10 isolates of 6M, 11M, 15W, 146M, 178W, 200W, 151M, 154M, 178W and 45W. The third group consists of 8 isolates 237M, 262M, 51W, 74M, 34M, 25M and 81W. The final group consist of 2 isolates 71W and 95M.

Within each group, members share at least 90% of the PCR products Table (3).

Table (3): Grouping of *Saprolegnia* species according to the average similarity of isolates based on Random amplification of polymorphic DNA (RAPD).

Group number	Members	Similarity %
Group 1	32M,172W,245M,247M,248M,22W,1W,2W,3M,4M,6M,7M,8M,9M,10M,12W,13W,15M,17M,25W,48W,70M,98M ,195W,210W	90
Group 2	246M,11M,15W,146M,178W,200W,151M, 154M ,178W,45W	95
Group 3	237M,262M,51W,58W,74M,34M,25M,81W,63M	93
Group 4	71W,95M	98

Discussion

RAPD profiles obtained in this study revealed a genetic variation among *Saprolegnia* isolates from carp fish isolates and from water, eggs and fry from both hatcheries and separated the isolates into four different groups, such variables lead to separate the isolates to four groups, group 1 contained isolates, indicating that the group of closely related strains. Remaining carp fish isolates could be divided into group 2 group 3 and group 4.

This spread of a group of closely related strains came in agreement with [19] whom compared different isolates of *Saprolegnia parasitica* from trout by the RAPD-PCR technique and demonstrated that a group of closely related strains were spread over a large area and isolated from each other in well-separated water systems.

It is likely that the spreading of *Saprolegnia* sp. from pond to pond at one farm may be caused by farming management and the spreading from farm to farm may be due to birds, which feed on the fish. Many pathogenic strains of *Saprolegnia* spp. isolated from fish lesions apparently lack a sexual stage [20]. One would assume that asexual reproduction or sexual propagation in homothallic strains may lead to clonal propagation, i.e. a single genotype becomes widely spread. This may explain the high degree of homology within *Saprolegnia* species strains on a farm. [21] assessed pathogenic and genotypic variation in *Aphanomyces euteiches* expressed through asexually reproduced zoospores they noted that the small RAPD pattern variations within single-zoospore progenies suggest that some genetic changes might occur in *A. euteiches* during asexual reproduction. Therefore it is possible that the genetic diversity observed between Carp fish *Saprolegnia* species isolates within each group, of a single pond, may be caused by events during the asexual phase of reproduction (such as mitotic cross-over, mutations or somatic recombination).

Highly variable parasite populations, in general, are better adapted to changes in environmental conditions than those with little variation are. The presence of at least four very distinct groups or strains of *Saprolegnia* species in Iraqi hatcheries indicates that there are mechanisms to confirm that variable genotypes are spread in asexual *Saprolegnia*. Physiological properties of *Saprolegnia* have been used for the classification of different subgroups within a species. Studies with esterase isoenzyme patterns [20] and the relationships between growth rate and temperature [22] identified different groups of fish lesion isolates

These daughter colonies not only showed similar growth rate at original temperature as the parent strains, but they also exhibited an adaptation to high temperature. This event was suggestive of induction of cytoplasmic rather than a genetic change. In contrast, [22] studied daughter colonies of *Saprolegnia* isolates that stably expressed a slow growing

Phenotype and had suggested to be true genetic mutants, Possibly, our slowest growing isolates, 51W and 172W are stable mutants since repeated sub culturing did not affect their growth rates. From this study we recommended to focus on the molecular methods using specific primers for detecting

virulence factor and using Reverse Transcriptase PCR (RT-PCR) for isolation mRNA then forming cDNA then studying fungi behavior under certain conditions .

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