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Identification and Interrelationships Between *Saprolegnia* Species by RAPD Markers and Sequencing of D1/D2 Domain of 26S rDNA

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EIGHT SPECIES of the water mold *Saprolegnia: S. oblongata, S. hypogyna, S. ferax, S. furcata, S. parasitica, S. megasperma, S. prolifera* and *S. delica* were isolated from Danube, Vienna, Austria. The genetic variation and interrelationships between these species were studied using RAPD-PCR markers and the sequencing of the D1/D2 domain of the nuclear large subunit (26S) ribosomal DNA (rDNA). Only 28% out of 57 DNA bands generated by three informative primers (M13, V05 and V06) were common among the eight species of *Saprolegnia* reflecting high polymorphism (~72.0%). Specific RAPD markers were found for *S. delica, S. megasperma, S. parasitica, S. prolifera* and *S. furcata* while non markers were detected for *S. oblongata, S. hypogynae* and *S. ferax*. Cluster analysis indicated that the similarity between the tested species ranged from 65.4 to 86.3% and that *S. prolifera* and *S. delica* could be separated other species. The results UPGMA in conjunction with the unique DNA markers suggested that a RAPD approach allow the identification and discrimination of different species of *Saprolegnia*.

Sequences of the rDNA region (600 bp) were highly conserved (98.5% similarity) in the tested species of *Saprolegnia*. The differences in the 26S rDNA sequences (mostly due to transition mutations) were found between the tested species, except for *S.megasperma* and *S. furcata* and these two species displayed identical sequences of the 26S rDNA region. Both RAPD markers and the 26S rDNA sequences are powerful tools for the identification and discrimination of different species of *Saprolegnia*. However, the RAPD method, which relies on the fingerprints of fungal genomes is more sensitive and discriminative than 26S rDNA sequencing.

The genus *Saprolegnia* is found in fresh water habitats through out the world. This water mold is a member of the Oomycetes. Identification of freshwater fungal species by traditional microscope-based methods depends upon the ability of the species to sporulate and requires much expertise to distinguish between closely related species. Molecular techniques which detect DNA from all stages of the life cycle could potentially circumvent the problems associated with traditional methods of species identification (Nikolcheva *et al.*, 2003).

In recent years, numerous DNA-based fingerprinting methods that reveal genetic diversity among similar organisms have been developed. Random amplified polymorphic DNA (RAPD) analysis is a rapid technique and is widely used to assess inter- and intra-specific genetic variation at the molecular level using random amplified polymorphic DNA (RAPD) markers, produced by the polymerase chain reaction (PCR) (Williams *et al.*, 1990 and Welsh & McClelland, 1990). RAPD-PCR analyses have also been used to study the genetic differences between species, strains and isolates of different fungal genera such as *Saprolegnia* (Whisler, 1996), *Fusarium* (Altomare *et al.*, 1997 and Belabid *et al.*, 2004), *Pythium* (Tojo *et al.*, 1998), *Verticillium* (Perez-Artes *et al.*, 2000), *Aspergillus* (Megnegneau *et al.*, 1993). RAPD-PCR method was used by Whisler (1996) to determine the genetic variability among different isolates of *Saprolegnia hypogyna, S. diclina, S. ferax* and *S. parasitica.*

In addition to the utility of RAPD analysis in the discrimination of fungi at the species and subspecies levels, the examination of ribosomal DNA regions have also proved useful at strain, species or higher levels (Curran *et al.*, 1994). Sequencing of genes encoding for 18S and 26S ribosomal RNA (rRNA) as well as ITS-regions have been recently applied in fungal identification and discrimination (Kurtzman & Robnett, 1998; Petersen & Rosendahl, 2000; Fell *et al.*, 2000; Cappa & Cocconcelli, 2001; Scorzetti *et al.*, 2002 and Lopandic *et al.*, 2004).

The present investigation aimed to study the identification and interrelationships between eight species of *Saprolegnia* based on RAPD markers as well as the sequencing of the D1/D2 domain of nuclear large subunit (26S) ribosomal DNA (rDNA).

Material and Methods

Isolation of Saprolegnia species

This investigation was carried out during April to August, 2004 at the Institute of Applied Microbiology, University of Agriculture, Vienna, Austria. Water samples were collected during the spring and summer months of 2004 from the river Danube in sterile conical containing sterilized sesame seeds as baiting substance for fungi. For the recovery of *Saprolegnia* species, the baiting technique as described by Khallil (1984) was used. The seeded plates were incubated for two weeks at room temperature during which the zoosporic fungi which colonized the seeds were examined weekly. The species of *Saprolegnia* were identified according to Coker (1923) and Seymour (1970). Eight species of *Saprolegnia* were isolated namely; *S. oblongata, S. hypogyna, S. ferax, S. furcata, S. parasitica, S.megasperma, S. prolifera* and *S. delica.*

Isolation of DNA from fungal mycelium

The eight species of *Saprolegnia* were grown on glucose-peptone-agar medium (Willoughby and Pickering, 1977). For DNA extraction, a 1.0 cm square

disk of actively growing mycelium from each species was placed in 100 ml flasks containing glucose peptone medium (Lopandic *et al.*, 1996). The flasks were incubated at room temperature (20±2°C) for one week. Actively growing mycelium were harvested by filtration, washed with 0.1M $MgCl_2$ and dried on filter paper using vacuum. Total genomic DNA was extracted and purified according to Lopandic *et al.* (1996). The extracted DNA was checked on 1% agarose gels and used for RAPD-PCR and PCR-amplification of large-subunit (LSU) 26S ribosomal DNA (rDNA).

RAPD-PCR analysis

RAPD fingerprinting was carried out according to Lopandic *et al.* (1996). PCR reactions were conducted using 20 arbitrary primers (M13, V05, V06, BC04, BC05,BC06, BC07, BC08, BC09, BC10, BC11, BC12, BC13, BC14, BC15, BC16, BC17, BC18, BC19, BC20, Operon Tech., Inc.). Only three (M13, V05 and V06) out of these primers were successfully reacted with the DNA of the tested fungi. The primers M13 (5'GAGGGTGGCGGTTCT3'), V05 (5'TGCCGAGCTG3') and V06 (5'TGCAGCGTGG3') were used to study the interrelationships between *Saprolegnia* species. Amplifications were performed in 25 ml reaction buffer containing 38 µM $MgCl_2$, 100 mM dNTP, 0.1 mM primer, 0.55 units of *Taq* DNA polymerase. Each tube was overlaid with a drop of mineral oil. PCR was done in a Trio-Thermoblock TB1 thermocycler (Biometra company). A standard DNA Ladder (3000, 2000, 1500, 1200, 1081, 900, 800, 700, 600, 500, 400, 300 and 200 bp) was included on each gel.

Data analysis

Bright reproducible bands were binomially scored, 1 for presence and 0 for absence of bands. Nie and Li's coefficients of similarity (Nie and Li, 1979) were calculated on the resulting matrix. An unweighted pair-group arithmetic average (UPGMA) analysis performed on the matrix resulting from the Nie and Li's coefficient calculations was used to construct the dendrogram.

Partial sequencing of the gene coding for 26S rRNA

The D1/D2 domain of the nuclear large subunit (LSU 26S) ribosomal DNA (rDNA) region was amplified from the DNA of the 8 species of *Saprolegnia*. Two primers, NL1 (5'GCATATCAATAAGCGGAGGAAAA3') and NL4 (5'GGTCCGTGTTTCAAGACGG3'), were used for sequencing a fragment of approximately 600 bp (White *et al.*, 1990). The PCR was performed using following temperature program: 35 cycles of 96°C for 30s, 50°C for 15s and 72°C for 4 min with a final extension of 72°C for 4 min. Amplifications products were checked on 1% agarose gels. The PCR products were purified from the amplification mixture using milipore. Ultrafree-MC filters (Milipore Corporation, Bedford, MA) and used in cycle-sequencing reactions (ABI Dye Terminator Cycle Sequencing Ready Reactions Kit) together with the specific primers. Both strands of the 26S rDNA were sequenced on an automatic sequencer (ABI Prism TM 377 DNA Sequencer), and chromatograms of these

sequences were checked using Sequencer 3.1 (Gene Codes Corporations Inc., Ann Arbor). The partial sequences of 26S rRNA gene of the 8 species were compared with the published rDNA sequences obtained from Genebank (http://www.ncbi.nlm.nih.gov//).

Results

RAPD analysis

Identification of informative primers

Four *Saprolegnia* species (*S. delica*, *S. megasperma*, *S. furcata* and *S. parasitica*) were selected to identify primers that generate informative arrays of PCR products. Twenty oligonucleotide primers, which contained no palindromic sequences, were tested. The choice of the selected primers was based on the number of bands generated (with as few low-intensity bands as possible) as well as the quantity of different and reproducible patterns yielded. The primers M13 (5'GAGGGTGGCGGTTCT3'), V05 (5'TGCCGAGCTG3') and V06 (5'TGCAGCGTGG3') were selected because they satisfied the characteristics described above. A set of reproducible bands produced for a particular primer was defined as a "pattern."

Genetic variation and interrelationships between Saprolegnia species

The three selected primers were used to study the interrelationships between eight species of *Saprolegnia* (Fig.1). A total of 57 DNA bands were generated from all tested species with an average of 19 bands/primer (Table 1). Only 16 out of the 57 DNA bands (~28.0%) were common in all tested species while 41 (~72.0%) were polymorphic.

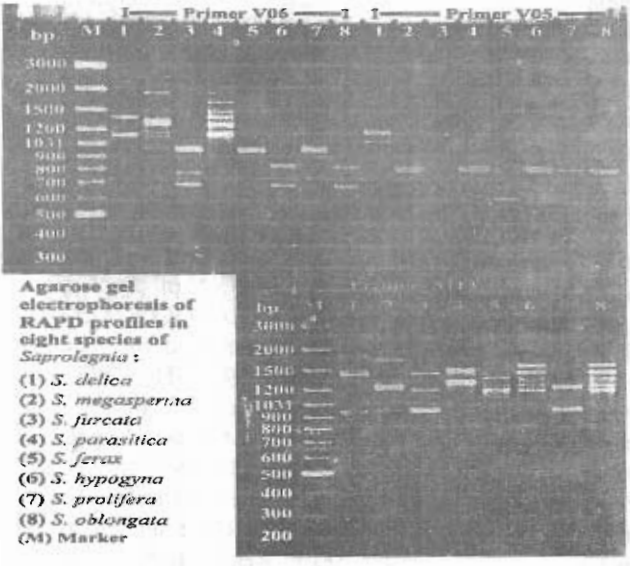

Fig. 1. Agarose gel electrophoresis of RAPD profile generated by the primers M13, V05 and V06 from 8 species of *Saprolegnia*.

TABLE 1. Survey of the RAPD-PCR markers in 8 species of *Saprolegnia* generated by the primers M13, V05 and V06.

No.	RAPD Markers Base pair	*S. delica*	*S.mega-sperma*	*S. furcata*	*S. para-sitica*	*S. ferax*	*S. hypo-gyna*	*S. prolifera*	*S.oblon-gata*
1	M13 2200	1	0	0	1	0	0	0	0
2	2100	0	1	0	0	0	0	0	0
3	2000	0	0	1	0	1	1	0	1
4	1900	1	0	0	1	1	0	0	0
5	1800	1	1	0	0	0	0	0	1
6	1700	0	1	0	0	1	0	0	0
7	1600	0	0	0	0	0	1	1	1
8	1500	1	1	1	1	1	1	1	1
9	1450	1	0	0	0	1	0	0	0
10	1400	1	1	1	1	1	1	1	1
11	1300	1	1	1	1	1	1	1	1
12	1200	1	0	1	0	1	1	0	1
13	1150	0	0	0	0	1	1	0	0
14	1100	1	1	0	0	0	0	0	0
15	1020	1	1	1	1	1	1	1	1
16	1050	1	1	1	1	0	0	0	0
17	900	0	0	1	0	1	0	0	0
18	870	1	0	1	1	0	0	0	1
19	750	0	0	1	0	1	1	0	1
20	700	1	0	0	0	1	0	0	1
21	600	1	0	0	0	0	0	0	0
22	500	1	0	0	0	0	1	0	0
23	V05 2800	1	0	0	0	0	0	0	0
24	2500	1	0	1	0	0	0	1	0
25	2200	1	0	0	0	0	0	1	0
26	2000	1	1	1	1	1	1	1	1
27	1800	0	0	0	1	0	0	0	0
28	1200	1	0	0	0	0	0	0	0
29	1030	1	1	1	1	1	1	1	1
30	900	0	0	0	0	0	0	1	0
31	800	1	1	1	1	1	1	1	1
32	750	1	1	0	1	0	0	0	0
33	700	0	1	1	1	0	0	1	1
34	600	1	1	1	1	1	1	1	1
35	530	1	0	0	0	0	0	0	0
36	420	0	0	0	0	0	0	0	1
37	400	0	0	1	0	0	0	1	0
38	250	0	0	1	0	0	0	0	0
39	V6 2700	1	0	0	1	0	0	0	0
40	2500	0	1	0	1	0	0	0	0
41	2300	1	1	1	1	1	1	1	1
42	2000	0	0	0	0	1	1	1	1
43	1950	1	1	1	1	1	0	1	0
44	1700	1	1	1	1	1	1	1	1
45	1500	1	1	1	1	1	1	1	1
46	1400	1	0	0	1	1	0	0	0
47	1350	0	1	0	0	0	0	0	0
48	1300	1	1	1	1	1	1	1	1
49	1200	0	1	1	1	1	0	0	0
50	1150	1	1	0	0	0	0	0	0
51	1100	1	1	1	1	1	1	1	1
52	1030	1	1	1	1	1	1	1	1
53	980	0	0	0	0	0	1	0	1
54	900	0	0	1	0	1	0	1	0
55	800	1	1	1	1	1	1	1	1
56	700	1	1	1	1	1	1	1	1
57	600	0	1	0	1	0	0	1	0

The presence of unique DNA bands in particular species could be used as positive DNA markers for each species and may be helpful in identification and discrimination of fungi. In the present investigation, the DNA bands of 1450 and 600 bp generated by M13 and the 2800 and 1200 and 530 bp bands generated by V05 were unique markers for *S. delica* (Table 1). The two bands of 2100bp (M13) and 1350bp (V06) were specific markers for *S. megasperma*. However, the DNA fragments 1800, 900 and 250bp generated by primer V05 were specific markers for *S. parasitica*, *S. prolifera* and *S. furcata*, respectively. Meanwhile, no specific markers were detected for *S. oblongata*, *S. hypogynae* and *S. ferax*.

The electrophoretic DNA banding patterns obtained from the 8 species of *Saprolegnia* were analyzed by using the MVSP computer software program of Nie and Li (1979) and clustered by unweighted pair group method based on arithmetic mean (UPGMA) as shown in Fig. 2. The UPGMA clustering analysis showed that all the tested species were clustered together in two groups with 65.4% genetic similarity. *S. parasitica*, *S. megasperma* and *S. delica* were clustered together in the second group with 70.8% genetic similarity, while the other 5 species were clustered in the first group at a genetic similarity of 73.4% (Fig.2). These results indicated the presence of clear-cut differentiating features between these two groups of *Saprolegnia* species at the molecular level.

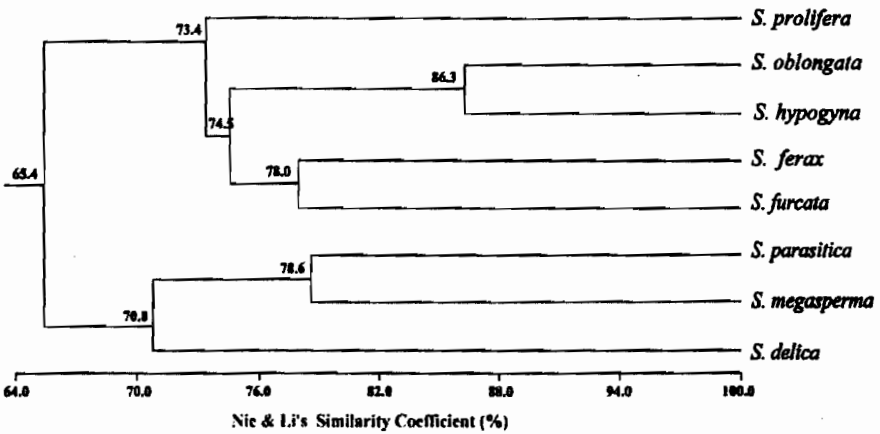

Fig. 2. Dendrogram demonstrating the interrelationships among 8 species of *Saprolegnia* based on the RAPD markers.

Results of RAPD profile analysis (Table 1 and Fig. 1) showed that each species possessed a distinct pattern of RAPD-generated PCR bands reflecting genetic differences between the tested species of *Saprolegnia*. The UPGMA analysis also revealed that the six species were separated in three subclusters (2 species/subcluster). In this instance, *S. oblongata* and *S. hypogyna* were clustered

together within 86.3% genetic similarity while, both *S. ferax* and *S. furcata* as well as *S. parasitica* and *S. megasperma* were separated in two clusters with ~78% similarity. These results clearly distinguished *S. oblongata* and *S. hypogyna* from *S. ferax* and *S. furcata* as well as from *S. parasitica* and *S. megasperma*. While, the dendrogram separated *S. prolifera* and *S. delica*, each in a single branch, from the other species.

Sequencing of 26S rDNA

The D1/D2 domain of the large subunit (26S) ribosomal RNA (rRNA) (600 bp) was sequenced from the eight species of *Saprolegnia* (Fig.3). The sequences were compared with the published rDNA sequences obtained from Genebank (http://www.ncbi.nlm.nih.gov//). Genebank contains, at present, the D1/D2 sequences of 13 species of *Saprolegnia*, including only 3 (*S. ferax, S. hypogyna* and *S. parasitica*) out of the 8 tested species (Fig.3). The results revealed that 98.5% of the 26S rDNA sequences were conserved in the 8 tested species of *Saprolegnia* and percentage was reduced to 87.35% when the 26S rDNA sequences of *Saprolegnia* species found in Genebank were taken into consideration.

Close similarity was found between the 26S rDNA sequence of *S. ferax* and that of Genebank entry AF218167Sfer (Fig.3). Minor differences (1.01%) in 26S rDNA sequences were observed between the isolated *S. hypogyna* and that of the Genebank (AF119613Shyp). All of these differences were due to transition mutations in 7 sites (4 between A, G and 3 between C, T) of the 600bp tested region. Meanwhile, transition, transversion and insertion mutations in 16 sites (2.7%) of the 26S rDNA sequences were found among the isolated *S. parasitica* as compared with that published in Genebank (AF218170Spar). Transitions were found at 14 sites (5 between A, G and 9 between C, T) while, transversion (G, T) was observed in only one site. Insertion of "G" was found at one site in the 26S rDNA sequences.

No information about the 26S rDNA sequences of *S. delica, S. oblongata, S. megasperma, S. furcata* and *S. prolifera* were available, at present, in Genebank. So the DNA sequences were compared with those of other species. These comparisons revealed the presence of genetic differences in 26S rDNA sequences between these species (Fig.3). All of these differences were due to transition mutations. In *S. delica*, 2 transitions between A,G and one between C,T were observed. While, 2 transitions between T,C and one between A,G were found in 26S rDNA sequences of *S. oblongata*. Only one transition (T,C) was observed in *S. prolifera*. Both *S. megasperma* and *S. furcata* possessed close similarity in the sequences of the 26S rDNA region.

Discussion

The RAPD-PCR technique requires only the presence of single "randomly chosen" oligonucleotide primers. The ability of RAPDs to produce multiple bands using a single primer means that a relatively small number of primers can

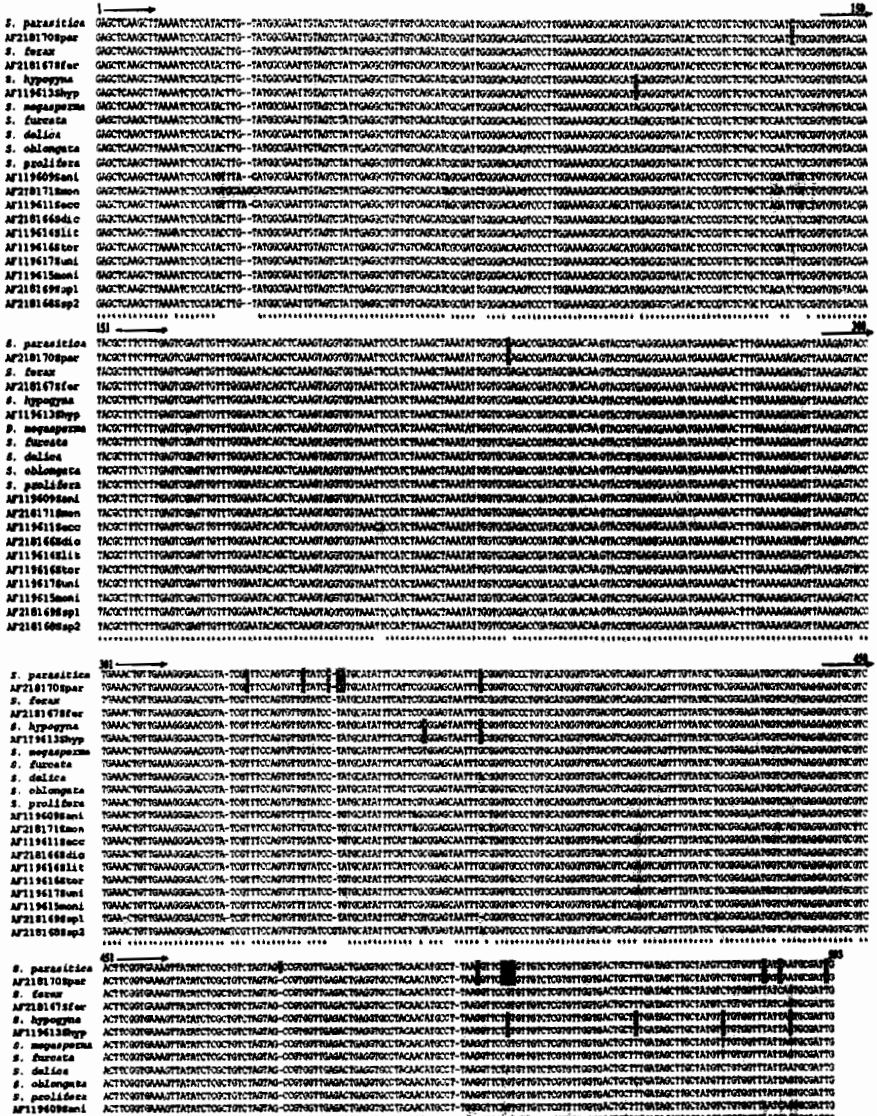

Fig. 3. Partial nucleotide sequencing of 26S rDNA region (600 bp) amplified from *Saprolegnia parasitica, S. ferax, S. hypogyna, S. megasperma, S. furcata, S. delica, S. oblongata* and *S. prolifera* in comparison to 13 species of Genebank. The codes of Genebank *Saprolegnia* species AF218170Spar, AF218167Sfer, AF119613Shyp, AF119609Sani, AF218171Smon, AF119611Secc, AF218166Sdic, AF119614Slit, AF119616Stor, AF119617Suni, AF119615moni, AF218169Ssp1 and AF218168Ssp2.

be used to generate a very large number of fragments. These fragments are usually generated from different regions of the genome and hence multiple loci may be examined very quickly (Edwards, 1998). In the present investigation, three informative primers (M13, V05 and V06) were analyzed by the RAPD-PCR technique to determine the genetic variability and the interrelationships between eight species of *Saprolegnia* isolated from the river Danube in Vienna. The three primers revealed high polymorphism (~72.0%) when reacted with the DNA of the 8 species. Such a high polymorphism reflects the high efficiency of the three primers in characterizing the different species of *Saprolegnia* at the molecular level. Similarly, Whisler (1996) used RAPD-PCR analysis and found high polymorphism (~88.0%) in different isolates and species of *Saprolegnia*. He also found that approximately 12% of the generated bands were common in all tested isolates and species of *Saprolegnia*.

The results also revealed the presence of unique DNA markers for *S. delica*, *S. megasperma, S. parasitica*, *S. prolifera* and *S. furcata*. The high level of polymorphism detected using RAPD's and the determination of unique DNA markers for 5/8 species of *Saprolegnia* suggested that the RAPD approach showed considerable potential for fungal species identification and discrimination. Similar conclusions were obtained in various fungal species (Megnegneau *et al.*, 1993; Whisler, 1996; Tojo *et al.*, 1998; Perez-Artes *et al.*, 2000 and Belabid *et al.*, 2004). The dendrogram showed that the similarity between the tested species ranged from 65.4 to 86.3%.

Whisler (1996) found that *Saprolegnia* isolates which showed least similarity in the UPGMA cluster analysis were distinguishable as different morphological species.

The UPGMA clustering analysis separated *S. prolifera* and *S. delica* from the other species. While, the other species were separated into three subclusters, as 2 species/subcluster. These results indicated that the species in the same subcluster are separated from those in the other subclusters. These results in conjunction with the unique DNA markers may help in the identification and discrimination of different species of *Saprolegnia*. Whisler (1996) used RAPD-PCR method to determine the genetic variability among different isolates of *Saprolegnia hypogyna, S. diclina, S. ferax* and *S. parasitica*. He reported that RAPD-PCR analysis supports the separation of *Saprolegnia* into the species *ferax, hypogyna* and *diclina.*

In the present investigation, the region of the 26S rRNA gene was highly conserved in the tested species of *Saprolegnia*. While, differences in the sequences of the 26S rDNA region, mostly due to transition mutations, were found between the tested species, except *S. megasperma* and *S. furcata*. However, the last two species displayed identical sequences of the 26S rDNA region, each of them has specific RAPD marker(s) and pattern. These results suggested that both RAPD and the 26S rDNA sequences are powerful methods for the identification and discrimination of different species of *Saprolegnia*. However, the RAPD method

that relies on the fingerprints of fungal genomes is more sensitive and discriminative than the 26S rDNA sequences. Megnegneau *et al.* (1993) studied the genetic variability and relatedness among Black Aspergilli strains and related species using RAPD method in comparison to rDNA and isozyme techniques. They suggested that the RAPD method is a quick and reliable tool for establishing the amount of genetic variability in closely related species. Lopandic *et al.* (2004) described two new species of *Trichosporon* using 26S rDNA, 18S rDNA and RAPD analysis. Sequencing of 18S and 26S rDNA as well as ITS-regions were used for identification and discrimination of various fungal species (Kurtzman & Robnett, 1998; Fell *et al.*, 2000; Cappa & Cocconcelli, 2001; Scorzetti *et al.*, 2002 and Lopandic *et al.*, 2004).

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تعريف وعلاقات القرابة بين أنواع فطر *سابروليجنيا* بواسطة واسماتRAPD وتحديد تتابعات مقطع D1/D2 في 26SDNA الريبوسومي

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تم عزل ثمانية أنواع فطرية تابعة لجنس *سابروليجنيا* من مياه نهر الدانوب عند مدينة فيينا بالنمسا. هذه الفطريات هى *سابروليجنيا ابلونجاتا* ، *س. هيبوجينا* ، *س.فيراكس* ، *س. فيوركاتا* ، *س. بارازيتيكا* ، *س. ميجاسبرما* ، *س. بروليفرا* ، *س. ديليكا*. تمت دراسة الاختلافات الوراثية وعلاقات القرابة بين هذه الأنواع الفطرية بواسطة واسمات RAPD وتحديد تتابعات مقطع D1/D2 لجزء الـ DNA الخاص بتحت الوحدة الكبيرة من الريبوسوم (26S rDNA). أوضحت النتائج أنه من بين ٥٧ حزمة نتجت من استخدام ثلاثة بادئات فعالة (M13, V-05, V-06) كان ٢٨% منها فقط مشتركا بين الأنواع الثمانية من فطر *سابروليجنيا* مما يعكس وجود نسبة عالية من التعدد المظهري لحزم الـ DNA الناتجة من هذه البادئات في الفطريات المختبرة. وجدت واسمات خاصة من RAPD مميزة للفطريات *سابروليجنيا فيوركاتا* ، *س. بارازيتيكا* ، *س. ميجاسبرما*، *س. بروليفرا* ، *س. ديليكا* بينما لم توجد واسمات مميزة لفطريات *سابروليجنيا ابلونجاتا* ، *س. هيبوجينا* ، *س. فيراكس*. أظهر التحليل العنقودي أن نسبة التماثل بين الفطريات المختبرة تراوحت من ٨٦,٣% إلي ٦٣,٤%. وقد فصل التحليل العنقودي كل من *س. بروليفرا* ، *س. ديليكا* عن الفطريات الأخرى. أوضحت نتائج التحليل العنقودي بالاتحاد مع وجود الواسمات الخاصة ببعض الفطريات أن تقنية الـ RAPD-PCR ذات أهمية كبري في التمييز بين الأنواع المختلفة لفطر *سابروليجنيا*.

أظهرت تتابعات 26S rDNA لمقطع طوله ٦٠٠ زوج من القواعد درجة عالية من المحافظة (٩٨,٥% تماثل) في الأنواع الثمانية من فطر *سابروليجنيا*. أما الاختلافات في تتابعات الـ 26S rDNA (معظمها يرجع إلي طفرات استبدال متناظر) قد وجدت في جميع الفطريات المختبرة فيما عدا *سابروليجنيا فيوركاتا* ، *س. ميجاسبرما* حيث اظهر هذان الفطران تتابعات متطابقة لمقطع 26S rDNA بينما تميز كل منهما بواسمات RAPD خاصة. أوضحت الدراسة أن تقنية الـ RAPD وتقنية تتابعات 26S rDNA تعتبر طرق فعالة في توصيف وتمييز الأنواع المختلفة لفطر *سابروليجنيا*. بينما تحقيق بصمة جينوم الفطريات بواسطة تقنية الـ RAPD يكون أفضل وأكثر تمييزا من تقنية تتابعات 26S rDNA.