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Prevalence of a single fish-pathogenic *Saprolegnia* sp. clone in Finland and Sweden

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ABSTRACT: Thirty-one isolates of *Saprolegnia* sp., most originating from infected salmon or trout, were characterised genetically and physiologically. The majority (6 of 31) of the isolates from several widely separated geographical locations was found to be genetically almost identical as assessed by RAPD-PCR. The remaining isolates belonged to 3 different groups with 1 to 3 representatives each. It is suggested that the first group of isolates represents a virulent form of the organism that has been widely spread by clonal propagation. The ability to repeated zoospore emergence, as an alternative to direct germination, seems to characterise specific *Saprolegnia* genotypes that may have adapted to certain hosts.

KEY WORDS: *Saprolegnia* · Saprolegniosis · Fish disease · Random amplification of polymorphic DNA RAPD · Repeated zoospore emergence

INTRODUCTION

During recent years, outbreaks of saprolegniosis, the disease caused by *Saprolegnia* spp., in freshwater fishes have become common in Scandinavia. These outbreaks have severely affected farmed salmonids, and have occasionally been encountered in fishes in natural freshwater bodies of Finland and Sweden. The gross sign of the disease typically displays as a relatively superficial, cotton-wool like, white growth of mycelia on the fish skin, especially around the head, dorsal and caudal fins, gills, in the muscular layer and internal organs (Khoo 2000, Grandes et al. 2001, Hussein et al. 2001). Even though *Saprolegnia* sp. could infect many fish species, outbreaks of saprolegniosis are frequently found among salmonid species and their eggs (Hatai & Hoshiai 1992, Noga 1993, Diéguez-Uribeondo et al. 1996, Bruno & Wood 1999, Hussein et al. 2001) and in farmed channel catfish *Ictalurus punctatus* (Durborow et al. 1991, Bly et al. 1992, Bangyeekhun et al. 2001).

Several species of *Saprolegnia* have been found to be pathogenic to fishes (Noga 1993). Taxonomical identification of *Saprolegnia* sp. using classical criteria is based on morphology of the reproductive structures, i.e. antheridia, oogonia and oospores (Willoughby 1978, Neish & Hughes 1980). However, many fish-pathogenic *Saprolegnia* isolates usually do not develop any sexual stages when cultured *in vitro* and therefore cannot be taxonomically classified (Beakes & Ford 1983, Grandes et al. 2000). Characterisation of fish-pathogenic *Saprolegnia* is useful for the furtherance of epidemiological studies of the source of infection, disease transmission, disease spreading and control of the disease. Some morphological and physiological studies have made it possible to classify different subgroups of *Saprolegnia* isolates. In an ultrastructural study of secondary cyst morphology, Grandes et al. (2000) distin-

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guished the fish lesion isolates from the water isolates, and Beakes (1983) was able to identify *S. parasitica* from *S. diclina*. Variation in esterase isoenzyme patterns (Beakes & Ford 1983) and differences in radial growth rate (Willoughby & Copland 1984, Hatai et al. 1990) have been used for determining distinct groups of fish lesion isolates. Restriction fragment length polymorphisms (RFLPs) are useful for classification of *Saprolegnia* and could distinguish *S. parasitica* from *S. diclina* (Molina et al. 1995). Also, random amplification of polymorphic DNA polymerase chain reaction (RAPD-PCR; Welsh & McClelland 1990, Williams et al. 1990) has been applied for analysis of the fish pathogenic *Saprolegnia* genome (Diéguez-Uribeondo et al. 1996, Bangyeekhun et al. 2001). This latter method provides a sensitive and rapid assay for the assessment of genetic distance between different isolates.

In the present study, we applied the RAPD-PCR technique and the presence or absence of repeated zoospore emergence to characterise *Saprolegnia* sp. isolates obtained from Finland and Sweden to investigate the epidemiology of the saprolegniosis in this region. We found that the majority of the isolates belonged to a single genetically defined group that probably has been widely spread by clonal propagation.

MATERIALS AND METHODS

***Saprolegnia* strains.** Thirty-one isolates of *Saprolegnia* spp. were isolated from infected tissue of the brown trout *Salmo trutta* m. *lacustris*, trout *S. trutta*, whitefish *Coregonus lavaretus*, rainbow trout *Oncorhynchus mykiss*, brook trout *Salvelinus fontinalis*, landlocked salmon *Salmo salar* m. *sebago*, salmon *S. salar*, noble crayfish *Astacus astacus*, and pond water from 6 different locations in Finland and 1 location in Sweden. The isolates from Finland are designated FinX, where X is the isolation number and the isolates from Sweden are designated Swe203 and Swe239 (see Table 1). The following reference isolates were used for comparison: *S. parasitica* (Spt) isolated from freshwater crayfish *Astacus leptodactylus* (Söderhäll et al. 1991), *S. parasitica* (Spt 198A) isolated from brown trout *Salmo trutta* (Diéguez-Uribeondo et al. 1996), *Saprolegnia parasitica* (Spt ACTT # 42062/ TP41; formerly named *S. diclina* Type 3 by Willoughby 1978), *Saprolegnia* sp. (CF91-1, CF96-2 and CF98-8) isolated from channel catfish (Bly et al. 1992, Bangyeekhun et al. 2001) and *S. diclina* (Sdi 2003h). All isolates were maintained on PG-1 agar medium (Unestam 1965).

Repeated zoospore emergence. Repeated zoospore emergence was performed according to the method described by Diéguez-Uribeondo et al. (1994b). Briefly, mycelia were grown in PG-1 drop cultures for 3 d at 20°C. To trigger sporulation, the mycelia were washed 3 times with sterile lake water and then incubated in petri dishes containing lake water for 14 h at 20°C to allow release of zoospores. The swimming zoospores were transferred to the test tubes, which then were agitated in a vortex mixer for 45 s to obtain synchronous encystment. The release of secondary zoospores was observed under the microscope after incubating the cyst suspension at 20°C for 150 min.

DNA extraction. A small piece of mycelium (approximately 1.5 mg dry weight) grown in PG-1 drop culture was inoculated in a 100 ml flask containing 25 ml PG-1 medium and cultured with shaking at 20°C. After 3 d, the mycelia were harvested, washed with sterile water, and ground in liquid nitrogen with a mortar. The total genomic DNA was extracted according to the method described by Lee & Taylor (1990). The DNA extracts were stored at –20°C until required.

RAPD-PCR. The PCR reactions were carried out in 25 µl volumes containing 100 ng genomic DNA, 10 mM Tris-HCl (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each of dNTPs (Amersham Pharmacia Biotech), 0.4 µM random primer, 1.5 Units of Taq DNA polymerase (GibcoBRL) and 1.5 Units of Platinum Taq Antibody (GibcoBRL). Nine nucleotide primers (Operon) were used: A04 (5′-AATCGGGCTG-3′), A07 (5′-GAAACGGGTG-3′), A09 (5′-GGGTAACGCC-3′), A10 (5′-GTGATCGCAG-3′), A12 (5′-TCGGCGATAG-3′), B01 (5′-GTTTCGCTCC-3′), B05 (5′-TGCGCCCTTC-3′), B11 (5′-GTAGACCCGT-3′), and B15 (5′-GGAGGGTGTT-3′). Each PCR reaction was run with a negative control containing no DNA. Amplifications were performed in a Perkin Elmer GeneAmp PCR system 9700 programmed for 1 cycle of initial denaturation at 94°C for 5 min, 45 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min, and a final extension at 72°C for 7 min. The PCR products were separated on 1.4% agarose gel, and 100 bp ladder DNA (Amersham Pharmacia Biotech) and lambda DNA/*Hin*dIII were used as molecular weight markers. Gels were stained with ethidium bromide, visualised by UV illumination and photographed.

Data analysis. RAPD-PCR was performed at least twice and only reproducibly amplified markers were scored as present (1) or absent (0). Similarity coefficients (F) between 2 isolates were calculated according to the formula of Nei & Li (1979): $F = 2N_{xy}/N_x+N_y$, where N_{xy} is the number of common fragments between 2 isolates, and N_x and N_y are the number of fragments in Isolate x and y, respectively. Pooled data from 9 primers were used for this calculation. The dendrogram was constructed from the similarity values obtained from pairwise similarity among all isolates as described by Fegan et al. (1993).

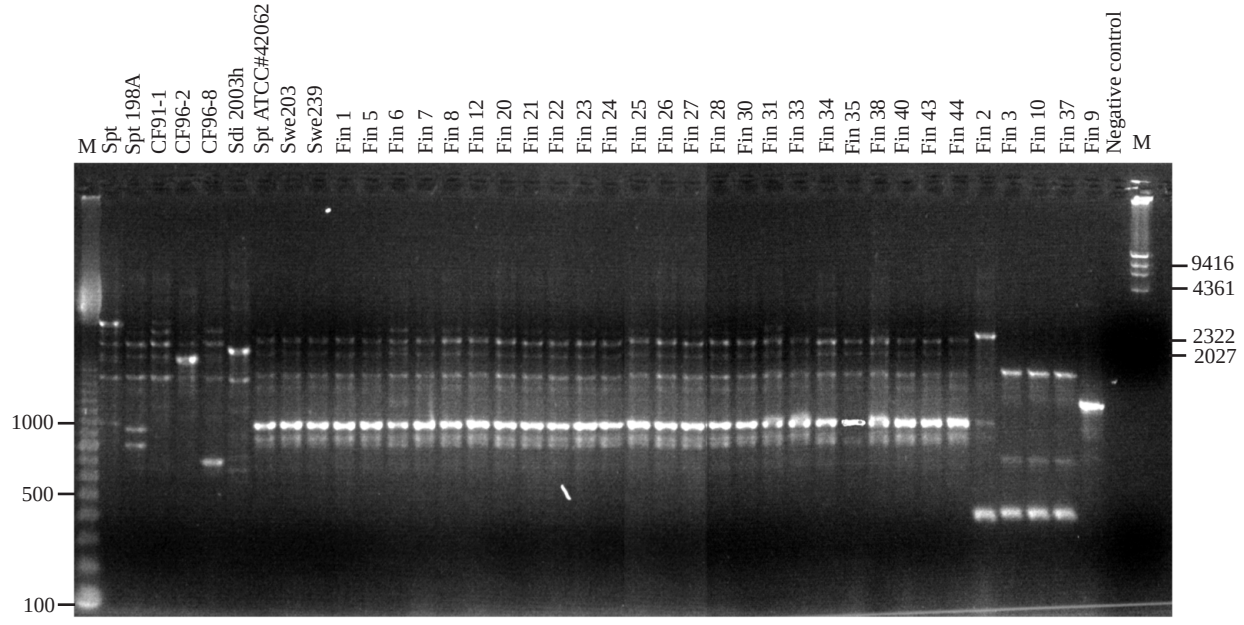

Fig 1. *Saprolegnia* spp. Amplification of genomic DNA from different isolates using Primer B15. M: molecular weight markers; 100 bp DNA ladder marker is on the left and lambda DNA/*Hin*dIII on the right. Abbreviations as in Table 1

RESULTS AND DISCUSSION

Characterisation of *Saprolegnia* sp. isolates based on RAPD analysis

Nine primers, which gave consistent results and produced a reasonable number of identifiable and polymorphic bands, were used for RAPD analysis. The amplification pattern of the PCR products from independent genomic DNA preparations of each isolate was consistent. Pooled data from 9 primers gave a total of 1010 clearly amplified PCR bands in 109 different positions, the average number of bands per isolate was 26.6. The size of the fragments produced ranged from 0.3 to 30 kb and there were 70 of 109 positions, i.e. 62.2%, with a product size larger than 1 kb. An example of an RAPD pattern generated by Primer B15 is shown in Fig. 1.

All PCR fragments obtained were used for genetic distance analysis. The dendrogram constructed from the pairwise similarity among all *Saprolegnia* sp. isolates demonstrates that the tested isolates of *Saprolegnia* sp. (Table 1) are genetically different from the reference isolates, except for Spt ATCC # 42062, and can be grouped into 4 distinct groups (Fig. 2). Within each group, members share more than 80% similarity of the RAPD band pattern; i.e. the average similarity of Group 1 was 95.9% and that of Group 3 was 100%. In addition, clusters of 100% identity were found; i.e. Cluster 1a: Fin 1, Fin 5, Fin 6, Fin 8, Fin 23 and Fin 28; Cluster 1b: Fin 20 and Fin 22; Cluster 1c: Fin 21 and Fin 26; Cluster 1d:

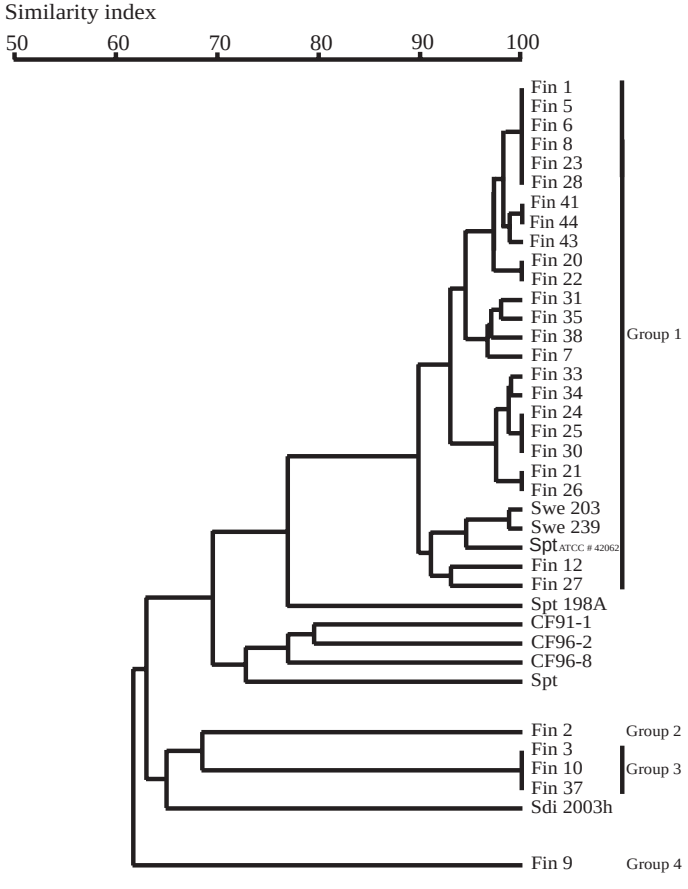

Fig 2. *Saprolegnia* spp. Dendrogram of 35 isolates based on RAPD dataset. Abbreviations as in Table 1

Fin 24, Fin 25 and Fin 30; Cluster 1e: Fin 3, Fin 10 and Fin 37; Cluster 3a: Fin 3, Fin 10 and Fin 37 (Fig. 2).

Group 1, the largest group, contained 26 of 31 isolates, which were sampled from several salmonid species or sources of pond water from 1995 to 2001, and Spt ATCC # 42062 (Table 1, Fig. 2). The spread of Group 1 is demonstrated by its presence in 6 of 7 examined locations at least 50 km apart (Fig. 3). Thus, a group of closely related strains has prevailed for over 7 yr in widely separated geographical localities, suggesting that its members are capable of causing persistent infections to fishes. It seems likely that Group 1 is dominant among isolates at Laukaa. It is interesting that a cluster of 100% similarity (Cluster 1a) was isolated from 4 geographically separated locations and in different years (Table 1). One of the reference strains, Spt ATCC # 42062, fell into Group 1. This strain is a pathogen of brown trout *Salmo trutta*, and was isolated from Cumbria, UK (Willoughby et al. 1983). This implies that the members of Group 1 occur in many areas, although more specimens from different areas must be examined to confirm this.

Table 1. *Saprolegnia* spp. Isolates used for RAPD-PCR analysis and their repeated zoospore emergence (RZE) performance. Germ: germination; Fin: Finland; Swe: Sweden; Spt: *Saprolegnia parasitica*; Sdi: S. diclina; CF: *Saprolegnia* sp.

RAPD group	Isolate	Location	Year of isolation	Source	% Germ or RZE		Zoospore generations
First clade							
Group 1	Fin1	Rautalampi	2000	*Salmo trutta* m. *lacustris*	Germ	95.4 ± 1.5	1
	Fin 5	Helsinki	1999	*Salmo trutta* m. *lacustris*	Germ	96.6 ± 0.9	1
	Fin 6	Laukaa	1999	Pond water	Germ	95.1 ± 0.6	1
	Fin 8	Laukaa	1999	*Coregonus lavaretus*	Germ	94.1 ± 0.6	1
	Fin 23	Laukaa	1999	*Salmo salar*	Germ	94.6 ± 1.2	1
	Fin 28	Paltamo	2000	*Salmo salar*	Germ	93.7 ± 1.3	1
	Fin 20	Laukaa	1998	*Salmo trutta* m. *lacustris*	Germ	93.2 ± 0.8	1
	Fin 22	Laukaa	1998	*Salmo trutta* m. *lacustris*	Germ	93.9 ± 1.0	1
	Fin 40	Tervo	2001	*Oncorhynchus mykiss*	Germ	95.3 ± 1.8	1
	Fin 44	Tervo	2001	*Oncorhynchus mykiss*	Germ	96.7 ± 1.2	1
	Fin 43	Tervo	2001	*Salmo trutta* m. *lacustris*	Germ	93.2 ± 0.9	1
	Fin 7	Paltoma	2000	*Salmo trutta* m. *lacustris*	Germ	95.7 ± 1.9	1
	Fin 31	Paltamo	2000	*Salmo trutta* m. *lacustris*	Germ	96.5 ± 2.0	1
	Fin 35	Paltamo	1996	*Salvelinus fontinalis*	Germ	96.2 ± 0.6	1
	Fin 38	Paltamo	2000	*Salmo trutta* m. *lacustris*	Germ	94.9 ± 0.8	1
	Fin 21	Laukaa	1998	*Salmo trutta* m. *lacustris*	Germ	91.2 ± 1.7	1
	Fin 26	Laukaa	2000	Salmo trutta	Germ	95.4 ± 0.9	1
	Fin 24	Laukaa	1999	*Salmo trutta*	Germ	92.9 ± 1.8	1
	Fin 25	Laukaa	1999	*Salmo trutta*	Germ	93.5 ± 2.1	1
	Fin 30	Laukaa	2000	*Salmo trutta* m. *lacustris*	Germ	95.3 ± 2.4	1
	Fin 33	Laukaa	2000	Pond water	Germ	97.9 ± 0.8	1
	Fin 34	Paltamo	1996	*Salmo salar* m. *sebago*	Germ	97.9 ± 0.7	1
	Fin 12	Laukaa	2000	*Salmo trutta* m. *lacustris*	Germ	94.4 ± 1.4	1
	Fin 27	Laukaa	2000	*Salmo trutta*	Germ	96.4 ± 2.9	1
	Swe 203	Umeå	1995	*Salmo salar*	Germ	94.4 ± 2.0	1
	Swe 239	Umeå	1995	*Salmo trutta*	Germ	95.0 ± 0.5	1
Ref. strain	Spt ATCC#42062	UK	1978	*Salmo trutta*	Germ	98.8 ± 1.3	1
Ref. strain	Spt 198A	Spain	1996	*Salmo trutta*	Germ	93.3 ± 2.0	1
Ref. strain	Spt	Turkey	1990	*Astacus leptodactylus*	RZE	98.2 ± 3.4	4
Ref. strain	CF91-1	USA	1991	*Ictalurus punctatus*	RZE	98.1 ± 2.9	4
Ref. strain	CF96-2	USA	1996	*Ictalurus punctatus*	RZE	98.4 ± 2.4	4
Ref. strain	CF96-8	USA	1996	*Ictalurus punctatus*	RZE	98.3 ± 4.1	4
Second clade							
Group 2	Fin 2	Paltoma	2000	*Salmo salar* m. *sebago*	Germ	94.3 ± 1.8	1
Group 3	Fin 3	Paltoma	1999	Pond water	RZE	94.1 ± 1.4	4
	Fin 10	Paltoma	1999	Pond water	RZE	95.5 ± 0.6	4
	Fin 37	Temmesjok	1999	*Astacus astacus*	RZE	94.4 ± 1.0	4
Ref. strain	Sdi2003h	UK		Saprophyte	Germ	94.6 ± 2.3	1
Third clade							
Group 4	Fin 9	Rautalampi	2000	*Salmo salar*	RZE	92.8 ± 2.3	4

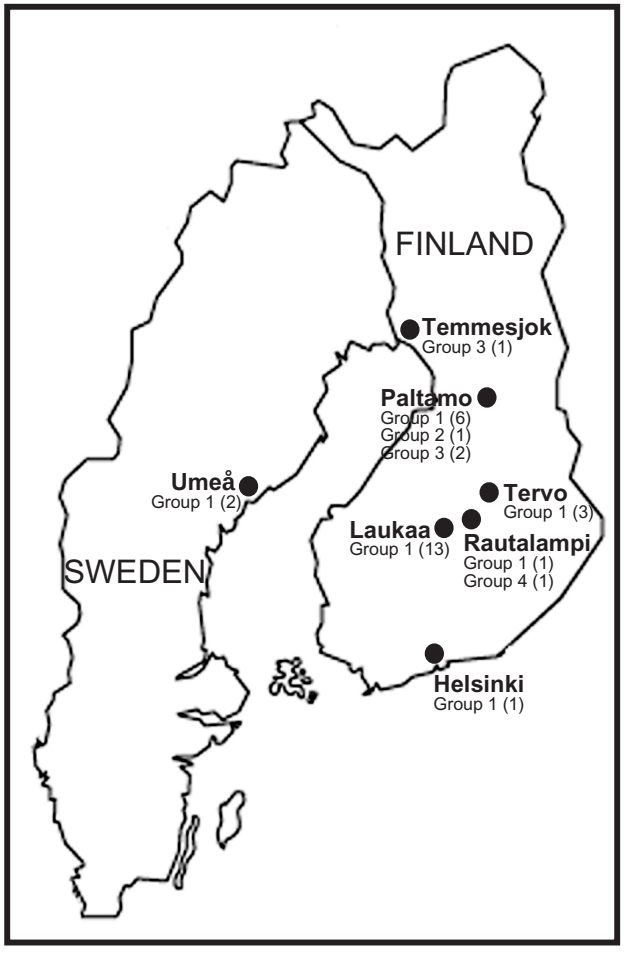

Fig 3. Map of Finland and Sweden showing location of sampling and distribution of *Saprolegnia* sp. isolates. Number in parentheses indicates number of isolates in each group (see Table 1)

Groups 2, 3 and 4 were minor groups containing 1 or 3 *Saprolegnia* sp. isolates per group. Group 2 was represented by a single isolate of Fin 2. Group 3 consisted of Fin 3, Fin 10 and Fin 37 with 100% similarity, whereas Group 4 was represented by Fin 9. Group 2 and Group 4 were obtained from infected fishes at Paltamo and Rautalampi, respectively. The members of Group 3 were isolated from pond water at Paltamo (Fin 3 and Fin 10) and from noble crayfish *Astacus astacus* at Temmesjok (Fin 37), demonstrating at least a limited spread of this genotype. Söderhäll et el. (1991) isolated *S. parasitica* (Spt) from a Turkish specimen of the freshwater crayfish *A. leptodactylus* that was able to infect and kill 3 different species of crayfishes, i.e. *A. astacus*, *Pacifastacus leniusculus* and *Procambarus clarkii* (Diéguez-Uribeondo et al. 1994a). This Spt isolate was also included in this study as one of reference strains. The RAPD analysis indicated that Spt and Fin 37 are different genotypes. Therefore, this study indicates that there are at least 2 genotypes of *Saprolegnia* that may parasitize crayfishes although *Saprolegnia* spp. are so far known only as severe pathogens of fishes.

Except for Sdi2003h, the isolates in this study did not reproduce sexually in the laboratory, and thus could not be distinguished to species level. Therefore, it is possible that the 4 distinct RAPD groups do not belong to the same species. Based on RAPD analysis, the dendrogram clearly separates the isolates into 3 different clades (Fig. 2), with the first clade composed of Group 1, Spt, Spt ACTT # 42062, Spt 198A and catfish isolates, the second clade of Groups 2 and 3 and Sdi 2003h, and Fin 9 constituting a third clade. Hence, we propose that the closely related strains of Group 1 are plausibly *Saprolegnia parasitica* and the closely related strains of Groups 2 and 3 may be *S. diclina*.

Applying the RAPD-PCR technique, Diéguez-Uribeondo et al. (1996) demonstrated the spread of a group of closely related strains of Spanish *Saprolegnia parasitica* isolated from infected trout, and Bangyeekhun et al. (2001) showed the presence of 3 genetically distinct groups of catfish isolates of *Saprolegnia* sp. in Mississippi. In this study, we included the Spanish *S. parasitica* (Spt 198A) and 3 isolates of American *Saprolegnia* sp. (CF91-1, CF96-2 and CF96-8; 1 isolate of each group) to compare with Finnish and Swedish *Saprolegnia* sp. isolates. The RAPD analysis showed that the genetic similarity of both Finnish and Swedish isolates to Spanish and American isolates is less than 80% (Fig. 2). The genotype variation among *Saprolegnia* sp. isolates observed in our study also suggests that there is genetic dissimilarity of fish pathogenic *Saprolegnia* from different geographic locations, i.e. Northern Europe, Southern Europe and USA.

Repeated zoospore emergence

Repeated zoospore emergence is the capacity of an encysted spore to release a new zoospore generation instead of germinating. The members of Groups 3 and 4, and Spt CF91-1, CF96-2 and CF96-8 could produce about 90 to 95% secondary zoospores of the primary cysts, and these isolates could produce 4 generations of zoospores. However, the members of Groups 1 and 2, and Spt 198A, Spt ATCC # 42062 and Sdi 2003h underwent germination instead. In Isolate Spt 198A, we found that the germlings of encysted spores formed short hyphae, about 3 to 5 cyst diameters in length, and then terminated their growth. This result agrees with a previous report by Diéguez-Uribeondo et al. (1996). Cysts of zoospores from several species of Saprolegniales are able to release new generations of zoospores

instead of germinating. Cerenius & Söderhäll (1985) demonstrated that repeated zoospore emergence is a specific mechanism for a pathogenic *Aphanomyces* species and proposed this mechanism to be an adaptation to a parasitic mode of life. Several isolates of *Saprolegnia* sp. in this study and the trout isolates of *Saprolegnia parasitica* as reported by Diéguez-Uribeondo et al. (1996) did not exhibit repeated zoospore emergence. Thus, repeated zoospore emergence is not a specific phenomenon for fish-pathogenic *Saprolegnia* isolates since some pathogenic isolates exhibit germination after encystment. However, the result clearly indicates that repeated zoospore emergence is correlated to certain genotypes of *Saprolegnia* spp. The number of zoospore generations of Spt was shown to depend on the length of time the zoospore was allowed to swim (Diéguez-Uribeondo et al.1994b). It could produce 4 to 6 zoospore generations after zoospores of each generation were kept swimming for 15, 60 or 240 min, respectively. In this study, the zoospores of tested and reference isolates, which exhibited repeated zoospore emergence (including Spt), were allowed to swim for 150 min in each generation, and they could produce 4 zoospore generations. Hence, our study indicates that the relationship between the length of swimming time and maximal number of zoospore generations in *Saprolegnia* isolates is similar, although the isolates are genetically different (Table 1).

Variations of oogonium morphology (Willoughby 1978), esterase enzyme profiles (Beakes & Ford 1983) and cyst ornamentation (Grandes et al. 2000) have been used to distinguish subgroups of *Saprolegnia parasitica*. In this study, a relationship between zoospore behavior and host-specificity was observed. Except for Fin 9, the zoospores of salmonid isolates exhibit germination, whereas the zoospores of non-salmonid (catfish and crayfish) isolates perform repeated zoospore emergence (Table 1).

In summary, RAPD analysis suggested that there are at least 4 groups of *Saprolegnia* playing a pathogenic role in saprolegniosis in Scandinavia. The dominance of Group 1 over a large geographical area implies that it may represent a high virulent clone of *Saprolegnia*. Although repeated zoospore emergence is clearly not specific for pathogenic *Saprolegnia* spp., it is present in certain groups of isolates defined by genotype and host-specificity, and can be useful in distinguishing subgroups of pathogenic *Saprolegnia*. The virulence of the different subgroups remains to be clarified.

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