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Pulsed Field Gel Electrophoresis of Three Microsporidian Parasites of Fish

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Summary. The technique of pulsed field gel electrophoresis has been used to differentiate the chromosomes of *Glugea stephani*, *Microgemma ovoidea*, and two isolates of *Spraguea lophii* from different regions of Spain (Atlantic Ocean at Galicia and Mediterranean Sea at Catalonia). Although the karyotype of *M. ovoidea* could not be completely resolved, the range of sizes of its chromosomes was obtained (282-2601Kb). *G. stephani* showed 15 bands ranging 340-2654Kb and an estimated total size of the haploid genome of about 16775Kb. Both isolates of *S. lophii* had a very similar karyotype with 10 bands for the Atlantic isolate (range 266-1076 Kb) and 11 bands for the Mediterranean isolate (range 271-1120Kb). Digital processing of the gels allowed detection of non-homologous chromosome co-migration and revealed that both *S.lophii* isolates had 15 different kinds of DNA fragments, which could be interpreted by a homozygotic hypothesis (15 chromosomes) or a heterozygotic hypothesis (14 chromosome pairs, 13 pairs with the same size for both homologous, and a pair formed by a chromosome of about 423-490 Kb and its homologous of 353-495Kb).

Key words: DNA, *Glugea stephani*, heterozygosis, *Microgemma ovoidea*, PFGE, *Spraguea lophii*.

INTRODUCTION

Few studies of the microsporidian genome have been undertaken. The ribosomal RNA sequences have been the most studied field, especially for use in molecular phylogenies (Vossbrinck *et al*. 1987, 1993; Vossbrinck and Woese 1989; Hartakeerl *et al*. 1993; Malone and McIvor 1993; Zhu *et al*. 1993a, b, c, d; 1994). However, one of the perspectives for the future is the potential of those sequences for in vitro hybridization diagnosis (Zhu *et al*. 1993a).

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Despite this relative availability of sequence data there is little information on the organization of the microsporidian genome. This is due to the fact that microsporidian cells divide by cryptomitosis and therefore the different chromosomes cannot be seen as they can be seen in higher eukaryots during cell division. However the technique of pulsed field gel electrophoresis (PFGE) has permitted the separation of very large DNA fragments, so that chromosomes can be counted and sized. These are called molecular karyotypes.

Various molecular karyotypes can be found in the literature: *Nosema furnacalis, Nosema pyrausta* (Munderloh *et al*. 1990), *Vavraia oncoperae*, *Vairimorpha sp.* and *Nosema costelytrae* (Malone and McIvor 1993), *Glugea atherinae* and *Spraguea lophii*

(Biderre *et al.* 1994), *Nosema locustae* studied by Street (1994) and *Nosema bombycis*, studied per Kawakami *et al.* (1994). Some other data are available in the literature on other aspects of the microsporidian genome such as the location of RNAr genes in the chromosomes of about 770Kb (Kawakami 1994). The ploidy of some species of *Vairimorpha* are also known, although the available data is based on observations made on different species of this genus (Canning 1988). Other techniques such as flow cytometry have been used to compare the relative DNA contents of different microsporidian spores (Amigó *et al.* 1994)

In this paper a study is presented on the molecular karyotypes of spores of the microsporidian parasites of fish, *Glugea stephani*, *M. ovoidea* and two isolates of *Spraguea lophii* (from fish of the Atlantic Ocean and the Mediterranean Sea).

MATERIALS AND METHODS

Organisms. *Microgemma ovoidea* (Thèlohan, 1895) Amigó *et al.* (1996) obtained from *Cepola macrophthalma* L. caught in the Catalan coasts (NW Mediterranean Sea); *Glugea stephani* (Hagenmüller, 1899) Woodcock (1904) from *Platichthys flesus* L. in the western coast of Denmark; *Spraguea lophii* (Doflein, 1898) Weissenberg (1976) from *Lophius piscatorius* L. in Galicia (NW Spain); and *S. lophii* from *Lophius budegassa* L. in the Catalan coasts (NW Mediterranean). Microsporidian cysts were taken from hosts and homogenized. Spore suspensions were purified from this homogenate by centrifugation in discontinuous Percoll gradients (Jouvenaz 1981) and finally washed by centrifugation in Ringer's medium.

Sample preparation. Samples for PFGE were prepared according to Biderre *et al.* (1994), thus, each sample was centrifuged until a pellet of spores was obtained and resuspended in 500 µl of PBS at 37°C. 500 µl of agarose 2,3% at 37°C were added to this suspension and blocks were prepared in 100 µl molds. Spore suspensions had previously been adjusted to obtain approximately 10^8 spores/agarose block. Once gelified, blocks were treated with a solution of hydrogen peroxide 3% for 20 min at 37°C to induce spore germination (Vavra and Maddox 1976). Under these conditions, and since the sporoplasm emerges from the spore coat via the polar tube, the microsporidian DNA is accessible with only membrane digestion: after induction of germination, the blocks were washed with PBS and finally kept at 50°C for 49 h in a lysis buffer EDTA (0.5M)/n-lauroyl sarcosine (1%)/Proteinase K (2mg/ml). After this digestion, the blocks were ready for analysis.

Electrophoresis. The electrophoresis was made with a gel of agarose 1% in TBE 0.5x (Tris buffer 0,05M, sodium borate 0.025M, EDTA 1mM; adjusted to pH 8,5) in a Hoeffer rotating gel with 3L of TBE 0,5x in a bath kept at 15°C, with a program of 48 h and pulses of 55-95 s. In order to estimate the size of DNA fragments, a preparation of *Saccharomyces cerevisiae* (Strain S28C) was analyzed in parallel. Once finished, the electrophoresis gels were stained with ethidium bromide 0.4 µg/ml for 24 h and photographs obtained with an ultraviolet transilluminator (254 nm) and Polaroid film.

Digital analysis of gels. Prints of the obtained gels were digitallized with a scanner and analyzed using Imat software for electrophoresis gels developed by the Image Analysis and Process Unit of Scientific-Technical Services, University of Barcelona. This analysis permitted automatic recognition of bands, which were lately confirmed manually, giving a very precise location of the band front. After recognition of standard sample location, the program determines the best regression line relating the logarithm of distance and size in Kb, while also making fine adjustments for the trapezoid geometry of the gel.

After this step the program gave the sizes of each DNA band in the gel. Additionally, a quantitative densiometric analysis was made on each band to detect the possible occurrence of multiple bands as a consequence of the presence of non-homologous chromosomes of similar sizes. For this analysis the profiles of intensity of the different samples were obtained by calculating the surface corresponding to each band. Once each peak area was obtained, these were divided by the corresponding size in Kb. An average of four clearly simple bands was made to obtain a common denominator for a quotient that would give the number of fragments for each band (Fig. 1, Table 2)

RESULTS

Of the four analyzed isolates, bands and sizes were resolved for three: both *Spraguea lophii* isolates and *Glugea stephani* (Table 1). *Microgemma ovoidea* could not be resolved, although the range of sizes of chromosomes was obtained. (Fig. 1)

Table 1. Bands/sizes for the isolates of *Glugea stephani* and *Spraguea lophii*

Band number	Size (Kb)		
	Glugea stephani	*Spraguea lophii* (Atlantic isolate)	*Spraguea lophii* (Mediterranean isolate)
1	2654	1076	1120
2	2402	887	915
3	1990	767	796
4	1807	679	607
5	1340	490	510
6	1237	440	453
7	1022	388	423
8	918	353	374
9	690	301	314
10	607	266	295
11	539	271	
12	432		
13	418		
14	379		
15	340		

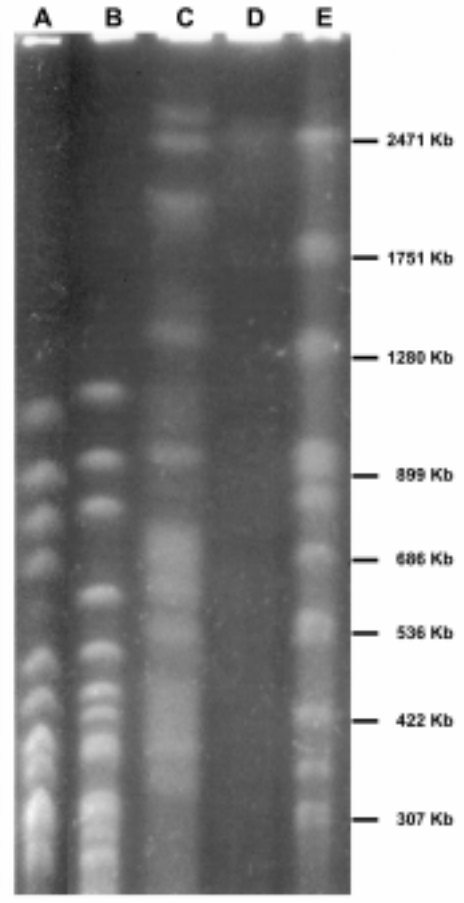

Fig. 1. PFGE of the different samples. A - *Spraguea lophii* (Atlantic); B - *Spraguea lophii* (Mediterranean Sea); C - *Glugea stephani*; D - *Microgemma ovoidea*; E - *Saccharomyces cerevisiae* S28C. Markers correspond to sizes of column E (Sample A had its position and contrast modified in this image to improve visibility)

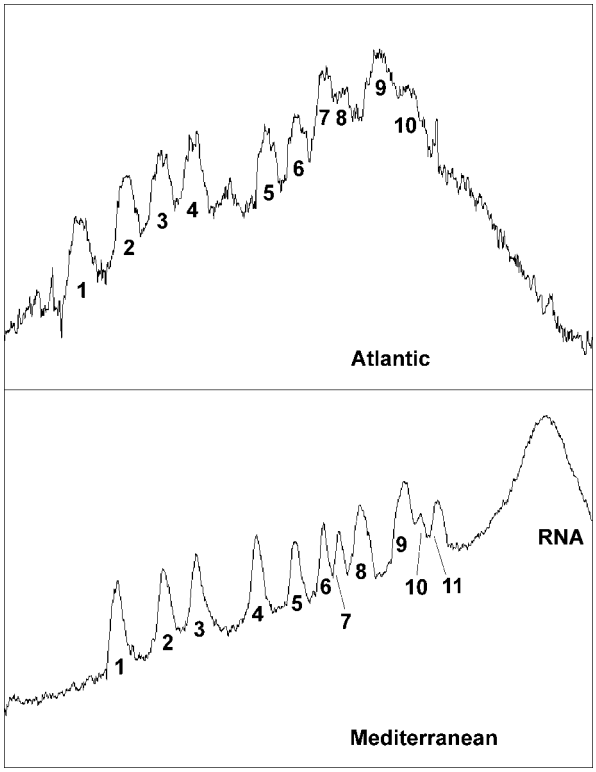

Fig. 2. Profiles of fluorescence intensity of the PFGE of both *Spraguea lophii* isolates

Glugea stephani showed a karyotype with poorly defined bands that could be resolved with a combination of digital analysis techniques and improved photo enhancements. This organism showed 15 bands distributed in a range of 340-2654Kb, giving a total size of the haploid endowment of approximately 16775Kb. Given the poor intensity of bands, no quantification was made for the detection of non-homologous chromosome co-migration.

Atlantic *S. lophii* showed a karyotype with clearly defined bands which was easily resolved with digital image processing (Figs 1, 2). This isolate showed 10 bands in a range of 266-1076 Kb. Naked eye observation of the gel suggested bands 7, 9 and 10 were multiple and the digital analysis based on intensity profiles suggested doublets for bands 7 and 10 and a quartet for band 9. This digital analysis also showed that band 8 had a relative endowment of 0.5 and band 5 a relative endowment of 1.5, which suggested that two homologous chromosomes had different sizes thus migrating to bands 5 and 8. This result suggests heterozygosis in this isolate. Multiple band analysis suggests that the total size of haploid endowment of the Atlantic isolate of *S. lophii* would be of 7272 Kb if the heterozygotic hypothesis is considered, or 7204 Kb if this heterozygotic hypothesis is not considered.

The Mediterranean isolate of *S. lophii* showed an exceptionally clear karyotype with 11 bands very clearly defined in the range of 271-1120 Kb (Figs 1, 2). Digital analysis of the gels showed the composition of multiple bands; thus bands 8 and 11 were doublets, and band 9 a triplet. Migration of homologous chromosomes was also detected with digital analysis of bands 7 and 10. With the homozygotic hypothesis this would then give a haploid

Table 2. Quantitative study of the bands of both *Spraguea lophii* isolates. The weighted number of bands (E) is the result of the division of the column surface/size (C) by the average surface/size of the simple bands (D)

	Spraguea lophii (Atlantic isolate)				Number of fragments	
Band number	(A) size	(B) Surface	(C) Surface/Size	(E) Weighted number of bands	Homozygosis hypothesis	Heterozygosis hypothesis
1	1076	821	0.763	0.92	1	1
2	887	701	0.790	0.95	1	1
3	767	598	0.780	0.94	1	1
4	679	620	0.913	1.10	1	1
5	490	555	1.133	1.37	1	1.5
6	440	394	0.895	1.08	1	1
7	388	691	1.781	2.15	2	2
8	353	183	0.518	0.63	1	0.5
9	301	976	3.243	3.91	4	4
10	266	490	1.842	2.22	2	2
(D) Average surface/size of simple bands (1, 2, 3, 4, 6)			0.828			
	Spraguea lophii (Mediterranean isolate)				Number of fragments	
Band number	(A) size	(B) Surface	(C) Surface/Size	(E) Weighted number of bands	Homozygosis hypothesis	Heterozygosis hypothesis
1	1120	1210	1.080	0.88	1	1
2	915	1012	1.106	0.90	1	1
3	796	1065	1.338	1.09	1	1
4	607	871	1.435	1.17	1	1
5	510	662	1.298	1.06	1	1
6	453	495	1.093	0.89	1	1
7	423	365	0.863	0.70	1	0.5
8	374	929	2.484	2.03	2	2
9	314	1048	3.338	2.72	3	3
10	295	528	1.790	1.46	1	1.5
11	271	672	2.480	2.02	2	2
(D) Average integral/size of simple bands (1, 2, 3, 4, 5, 6)			1.225			

endowment of 7351 Kb while the heterozygotic hypothesis would give a haploid size of 7287Kb for this isolate.

DISCUSSION

The protocol used for the preparation of samples has given optimal results for *S. lophii* isolates but poor results for *G. stephani* and quite bad results for *M. ovoidea*. This difference in the efficiency of the method might be due to the germination rate of each isolate being 4% in the case of *M. ovoidea*, 11% *G. stephani* and 40-50% in *S. lophii*. Furthermore it must also be considered that each isolate of *S. lophii* had been obtained from one cluster of cysts. This procedure not only reduces the heterogeneity of the genomic structure within the isolate but, given the clarity of gel results, suggests a clonal origin of each cluster of

Table 3. Comparison of known microsporidian molecular karyotypes

Species/isolate	Number of bands	Number of chromosomes	Range of sizes (Kb)	Estimate of genome size (Kb)	Reference
Nosema pyrausta	13	-	1390-440	10240	Munderloh *et al.* (1990)
Nosema furnacalis	13	-	1360-440	10240	Munderloh *et al.* (1990)
Nosema locustae	18	-	651-139	5364	Street (1994)
Nosema bombycis	18	-	1500-380	15330	Kawakami *et al.* (1994)
Nosema costelytrae	min 8	-	1810-290	7420	Malone and McIvor (1993)
Vairimorpha sp.	min 8	-	720-1790	9250	Malone and McIvor (1993)
Vavraia oncoperae from "grass grubs"	14	-	1930-130	8000	Malone and McIvor (1993)
Vavraia oncoperae from *Wiseana* sp.	16	-	1830-140	10240	Malone and McIvor (1993)
Glugea atherinae	16	-	2700-420	19510	Biderre *et al.* (1994)
Spraguea lophii from *L. piscatorius* (Atlantic)	12	-	980-230	6220	Biderre *et al.* (1994)
S. lophii from *L. piscatorius* (Atlantic)	10	15	1076-266	7272	Present work
S. lophii from *L. budegassa* (Mediter.)	11	15	1120-271	7287	Present work
Glugea stephani	15	-	2654-340	16775	Present work
Microgemma ovoidea	-	-	2601-282	-	Present work

cysts of *S. lophii*. In the case of *G. stephani* and *M. ovoidea*, clusters do not occur and this procedure of isolation cannot be followed, so the bands tend to be wider and loosely defined since homologous chromosomes of different individuals might show slight differences in size. In this sense, in *G. stephani* isolates there seems to be a dominant haplotype shown by the most clearly defined bands and other minority haplotypes shown as badly defined bands or shadows around the better defined ones.

Comparison of the karyotypes obtained in this study shows that there are clear differences in size of genome and range of chromosome size of *G. stephani* and *S. lophii*, which is not so surprising given the fact that both organisms belong to different genera with very different life and nuclear cycles. Comparison of data presented here with those of other authors shows that *G. stephani* has an endowment quite similar in size and range to *Glugea atherinae*. Furthermore, the results from *S. lophii* are very similar to those presented for the same organism by Biderre *et al.* (1994), despite the fact that the total estimated size is now a bit higher given the results of the digital analysis of gels. A comparison of the literature available on karyotypes is provided on Table 3. In this table, trends can be observed: *Glugea* and *Vavraia* (closely related genera) isolates show karyotypes with a wide range (from hundreds to about 2000 Kb); while isolates from genera with diplokaryotic spores, e.g. *Nosema*, *Vairimorpha* and *Spraguea*, tend to have small genomes of about 7000-9000 Kb. The exception here is *Nosema bombycis* with a 15000 Kb genome.

Special mention must be made on the variability found in the molecular karyotype of *S. lophii*, since despite an observed uniformity in the total size of endowment and number of bands in the isolate studied, the chromosome size seems to change. Such differences cannot be attributed to geographical origin since the number of analyzed samples is too low and, furthermore, our two isolates seem to differ as widely as the isolate of Atlantic origin analyzed by Biderre *et al.* (1994). The heterozygotic hypothesis of karyotype composition of *S. lophii* would then be supported by this variability.

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