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Karyotype and Genome Size of *Nadelspora canceri* Determined by Pulsed Field Gel Electrophoresis

Harold P. AMOGAN[1], J. Patrick MARTINEZ[2], Lynda M. CIUFFETTI[2], Katharine G. FIELD[1] and Paul W. RENO[1, 3]

[1]Department of Microbiology, Oregon State University, Corvallis, USA; [2]Department of Botany and Plant Pathology, Oregon State University, Corvallis, USA; [3]Coastal Oregon Marine Experiment Station, Newport, USA

Summary. Pulsed field gel electrophoresis was used to characterize the genome of microsporidian *Nadelspora canceri*. *N. canceri* spores isolated from Dungeness crab (*Cancer magister*) and red rock crab (*Cancer productus*) both yielded a karyotype of 10 chromosome-sized DNA bands. Close inspection of the band patterns and band sizes showed a difference in genome size between the two isolates. Spore isolates from Dungeness crab had a genome size of 7.4 Mb while spore isolates from red rock crab had a genome size of 7.3 Mb. Previously characterized microsporidians have a genome size range of 2.9-19.5 Mb and a karyotype ranging from 7-18 chromosome-sized DNA bands. *N. canceri* falls well within the genome size range and karyotype number of known microsporidians. The difference in genome size observed between the two spore isolates indicates intraspecies chromosome-size polymorphism.

Key words: chromosome-size polymorphism, microsporidia, protists.

INTRODUCTION

Phylum Microsporidia consists of eukaryotic protists parasitic to vertebrates and invertebrates. Members of this phylum have 7-18 chromosome-sized DNA bands (Wittner and Weiss 1999). Within-species variations in chromosome number and size have been detected in the phylum (Malone and McIvor 1993, Biderre *et al.* 1998). Variations in chromosome number and size may imply plasticity of the genomes and clonal reproduction of isolates (Venegas *et al.* 1997).

The microsporidian *Nadelspora canceri* is a pathogen of Dungeness (*Cancer magister*) and red rock crabs (*Cancer productus*). Among sampled populations of Dungeness crabs, *N. canceri* has a prevalence of infection ranging from 0.4% (Bodega Bay, CA) to 41.4% (Tillamook Bay, OR) (Childers *et al.* 1996). *N. canceri* has the potential to affect crabs important to commercial and sport fishermen. The geographic distribution, prevalence of infection, and spore ultrastructure of *N. canceri* has been described (Olson *et al.* 1994, Childers *et al.* 1996). However, information describing *N. canceri's* genome is lacking and warrants investigation. Based on small subunit ribosomal DNA (SSU

Address for correspondence: Paul W. Reno, Coastal Oregon Marine Experiment Station, 2030 South Marine Science Drive, Newport, Oregon, 97365, USA; FAX number: 1-541-867-0138; E-mail: paul.reno@hmsc.orst.edu

rDNA) sequence analysis, the parasite was placed in Baker's *Icthyosporidium* group (Baker *et al.* 1995, Amogan 2004). To further characterize the organism, we estimated the karyotype and genome size of *N. canceri* by pulsed field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Collection of infected crabs

A Dungeness crab containing *N. canceri* was donated for this study by Dr. Robert Olson (Coastal Oregon Marine Experiment Station, Oregon State University, Newport, OR). A red rock crab containing *N. canceri* was donated by a recreational fisherman in Yaquina Bay, Newport, OR. Crabs were caught using baited crab pots and rings. Infected crabs were detected by examining for milky white or yellowish crab muscle tissue observed at the carapace-leg junction. Infection was confirmed by microscopic observation of *N. canceri* spores. Spore samples obtained from the Dungeness crab were identified as NC1 and spores obtained from the red rock crab were identified as NC2.

Spore purification

To purify spores from crab muscle tissue, the infected tissue was minced with a razor blade and homogenized using a Dounce homogenizer. The homogenized sample was filtered twice through cheesecloth to remove large fragments of muscle tissue. The filtrate was then centrifuged for 30 min at 1,000 g. A pellet was obtained and resuspended in 5 ml of deionized water. The sample was added to 30 ml of 70% Percoll (Sigma-aldrich, St. Louis, USA) and centrifuged for 1 h at 1,000 g. Crab muscle tissue sedimented to the bottom of the centrifuge tube, and also formed a thin band above the 70% Percoll. Percoll lying between the thin band and pelleted crab muscle tissue was used for subsequent spore purification. Approximately 24-26 ml of Percoll were removed from the centrifuge tube and diluted to 40% Percoll using deionized water. The diluted Percoll was centrifuged for 1 h at 1,000 g. A pellet was obtained and resuspended in 250 ml of deionized water. The 40% Percoll was centrifuged three more times to obtain a total of 1 ml of spores suspended in deionized water. Spore concentration was estimated using a hemacytometer. Sample NC1 had a spore count of 7.51×10^7 spores/ml and sample NC2 had a spore count of 8.64×10^6 spores/ml.

Preparation of plugs and DNA extraction

The spore samples were first centrifuged for 30 min at 10,000 g. The pellets were then resuspended in 500 ml of 0.2 M KCl (pH 12.0) for 30 min at 30°C to induce polaroplast release. Five hundred microliters of melted 1.6% low melting point agarose (IBI, New Haven, USA) were added to the germinated spore sample to give a final concentration of 0.8% agarose. The 0.8% agarose-spore mixture was immediately pipetted into several plug molds (Bio-Rad, Hercules, USA) and allowed to solidify for 10 min at 4°C.

To obtain spore DNA, the prepared agarose plugs were immersed in 2.5 ml of lysis buffer (1% lauryl sarcosine, 100 mM EDTA, 10 mM Tris at pH 7.8, and 1 mg/ml Proteinase K). The plugs were then incubated in a 50°C water bath for 48 h. After 48 h the lysis buffer was replaced with 3 ml of 500 mM EDTA and incubated overnight at 4°C. The 500 mM EDTA was then replaced with 50 mM EDTA and the plugs stored at 4°C until ready to run PFGE. Crab muscle tissue from one uninfected Dungeness crab and one uninfected red rock crab were also subjected to the spore purification and spore lysis protocols. Agarose plugs prepared from these samples were used as negative controls.

Pulsed field gel electrophoresis conditions

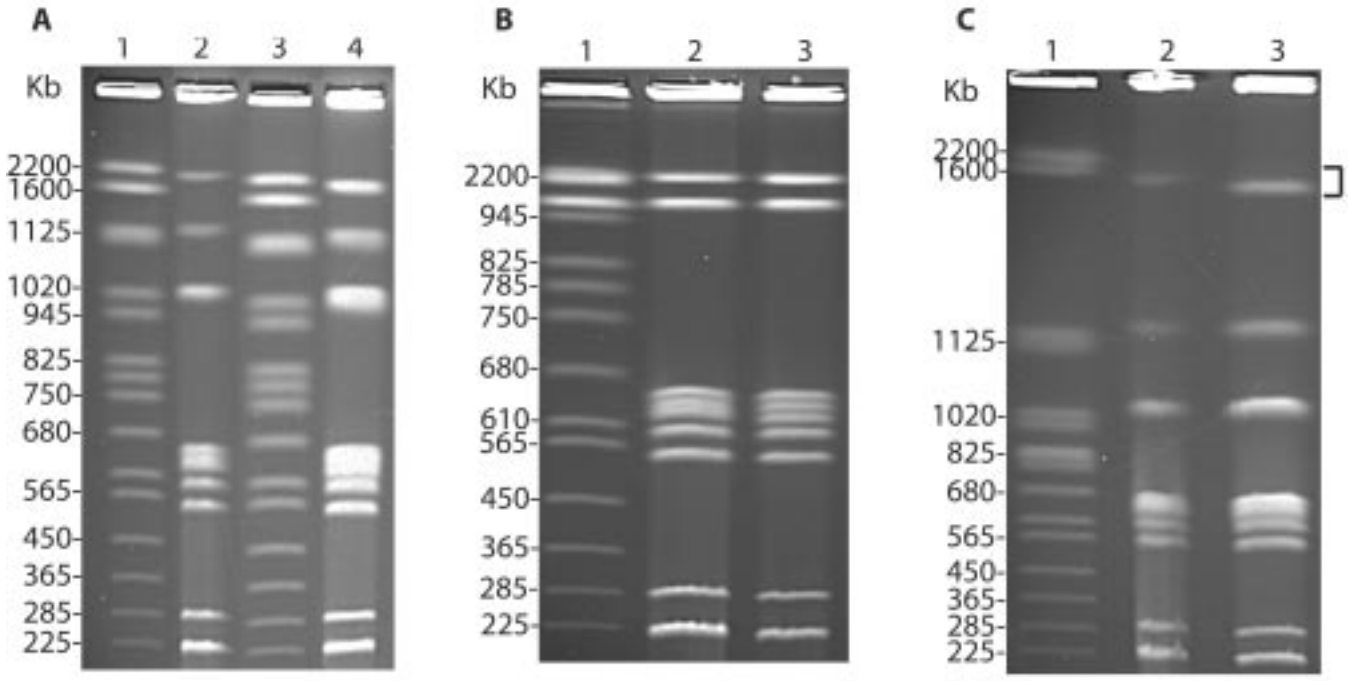
Five different PFGE conditions were used to resolve the chromosome-sized DNA bands from samples NC1and NC2 (Table 1). Condition 1 was used to obtain a general profile of *N. canceri's* karyotype, Condition 2 was used to resolve DNA bands in the 565-680 kilobase (Kb) range, Condition 3 was initially used to resolve DNA bands in the 945-1,125 Kb range, Condition 4 was used to determine whether *N. canceri* had DNA bands greater than 2,200 Kb, and Condition 5 was used to determine whether *N. canceri* had DNA bands smaller than 225 Kb. The DNA bands were resolved on 1% agarose gels (1 g agarose in 100 ml 0.5X TBE) placed onto a contour clamped homogenous electric field apparatus (CHEF DR II) (Bio-Rad, Hercules, USA). Each PFGE condition was run at least twice with similar results obtained. Gels were stained with 0.5 mg/ml ethidium bromide for 30 min. The gels were rinsed twice in deionized water and results were recorded using a gel documentation device (UVP, Upland, USA). To estimate the size of the chromosome-sized DNA bands, standard curves were created based on distance traveled by the *Saccharomyces cerevisiae* DNA markers (Bio-Rad, Hercules, USA) run simultaneously on each gel.

RESULTS

Figure 1 shows the results from running Conditions 1, 2, and 3. Figure 1A shows the results from running Condition 1. In Fig. 1A, bands I and II were well resolved near the bottom of the gel, bands III to VII were grouped closely together, and bands VIII, IX, and X were well resolved near the top of the gel. Lack of resolution among bands V to VII indicated another set of PFGE conditions was needed to separate DNA bands in the 565-680 Kb range. Figure 1B shows the results from running Condition 2. Condition 2 was used to resolve DNA bands in the 565-680 Kb range (Table 1, Fig. 1B). For sample NC1, bands V and VI were still close together and running the gel for an additional 4 h did not improve resolution. The adjacent *S. cerevisiae* DNA marker showed good separation between bands that were 610 Kb and 680 Kb in size (Fig. 1B). The separation of DNA size markers in the 610-680 Kb range indicates Condition 2 was optimal for resolution of bands V to VII. Condition 2 not only resolved bands V to VII (Table 1, Condition 2), but also compressed bands VIII, IX, and X (Fig. 1B). The *S. cerevisiae* DNA

Table 1. PFGE conditions used to estimate karyotype and genome size of several *Nadelspora canceri* spore isolates.

	Condition 1	Condition 2	Condition 3	Condition 4	Condition 5
Switch Time (Ramp)	45-100 s	60 s	85-100 s	250-900 s	4.3-21.4 s
Voltage	200 V (6 V/cm)	200 V (6 V/cm)	200 V (6 V/cm)	100 V (3 V/cm)	200 V (6 V/cm)
Temperature	14°C	14°C	14°C	14°C	14°C
Time	24 h	24 h	24 h	50 h	24 h



Figs 1A-C. Agarose gels showing resolution of chromosome-sized DNA from *Nadelspora canceri*. **A** - resolution of chromosome-sized DNA using Condition 1. Lanes identified from left to right as numbers 1-4: (1) *Saccharomyces cerevisiae* DNA marker, (2) NC1; *N. canceri* spores obtained from Dungeness crab, (3) *S. cerevisiae* DNA marker, (4) NC2; *N. canceri* spores obtained from red rock crab. **B** - resolution of chromosome-sized DNA in the 565-680 Kb range using Condition 2. Minimal migration observed for DNA bands in the 945-2,200 Kb size range. Lanes identified from left to right as numbers 1-3:(1) *S. cerevisiae* DNA marker, (2) NC1, (3) NC2. **C** - agarose gel showing a difference in migration rate between band X of NC1 and band X of NC2 (indicated by a bracket). Result obtained using Condition 3. Lanes identified according to Fig. 1B.

marker in Fig. 1B indicated minimal migration and loss of resolution for bands in the 945-2,200 Kb size range (Fig. 1B).

In various pulsed field results (data not shown), band VIII appeared brighter and wider than the other bands and called into question whether the band was a singlet or a doublet. Various switch times to improve resolution in the 945-1,125 Kb range did not further resolve band VIII into more bands. However, using Condition 3 (Table 1), a difference in size between band X of NC1 and band X of NC2 was detected (Fig. 1C). The bands differed in size by approximately 81 Kb, suggesting variation in chromosome size between the two spore samples (Table 2).

A total of 10 chromosome-sized DNA bands were identified by PFGE. Use of conditions appropriate for resolution of *Hansenula wingeii* chromosomes (Table 1, Condition 4) showed no additional chromosomes greater than 2,200 Kb for either NC1 or NC2 (data not shown). Pulsed field conditions designed to

Table 2. Estimates of number and size (in Kb) of chromosome-sized DNA bands obtained from *Nadelspora canceri* using PFGE. (NC1: spores isolated from Dungeness crab, NC2: spores isolated from red rock crab). Average Band Size in Kb

Band Number	NC1	NC2
X	1879	1798
IX	1223	1172
VIII	978	943
VII	598	592
VI	575	575
V	555	552
IV	533	533
III	496	499
II	327	330
I	280	290
Total	7444 Kb	7284 Kb

further separate the smallest DNA band (Table 1, Condition 5) also failed to reveal any bands smaller than 225 Kb (data not shown). Observed band patterns for both NC1 and NC2 were repeatable under all PFGE conditions used. Also, agarose plugs generated from uninfected Dungeness and red rock crab muscle tissue yielded no DNA bands on a pulsed field gel (data not shown). The absence of crab DNA indicates the DNA bands observed in Fig. 1 are from the *N. canceri* samples.

DISCUSSION

The estimated genome size of characterized microsporidians ranges from 2.9-19.5 Mb. Insect-infecting microsporidians vary widely in their karyotype, chromosome size range, and genome size (Table 3). In contrast, the mammal-infecting microsporidians have a karyotype of 10-12 chromosome-sized DNA bands, and a genome size range of 2-3 Mb (Table 3). *N. canceri's* genome size of 7.3-7.4 Mb is well within the genome size range of known microsporidians. The estimated genome and chromosome size range of *N. canceri* is most similar to the microsporidian *Nosema costelytrae* (Table 3). Unlike *N. canceri* which is a crustacean-infecting microsporidian, *N. costelytrae* is an insect-infecting microsporidian parasitic to the grass grub *Costelytra zealandica* (Malone and McIvor 1993).

Based on pulsed field conditions listed in Table 1, *N. canceri's* karyotype was estimated to be 10 chromosome-sized DNA bands. Although PFGE was carried out using conditions that should have resolved DNA bands in the 220-2,200 Kb size range, some DNA bands may yet be unresolved due to co-migration. In particular, band VIII may consist of several DNA bands. The use of densitometry or telomere probes could help determine whether band VIII is a singlet or a doublet (Blunt *et al.* 1997, Amigo *et al.* 2002, Zhong *et al.* 2002).

Table 3. Estimates of chromosome number and genome size of microsporidians, with *Nadelspora canceri* shown in bold.

Species	Host	Karyotype	Chromosome size range (Kb)	Genome size (Mb)	References
Vairimorpha sp.	Insects	8	720 to 1,790	9.2	Malone and McIvor 1993
Nosema costelytrae	Insects	8	290 to 1,810	7.4	Malone and McIvor 1993
Nadelspora canceri	Crustacea	10	280 to 1,879	7.4	Present work
Nadelspora canceri	Crustacea	10	290 to 1,798	7.3	Present work
Encephalitozoon cuniculi	Mammals	11	217 to 315	2.9	Biderre *et al*. 1995
Encephalitozoon intestinalis	Mammals	10	190 to 280	2.3	Peyretaillade *et al.* 1998
Encephalitozoon hellem	Mammals	12	175 to 315		Peyretaillade et al., 1998
Spragea lophii	Fish	12	230 to 980	6.2	Biderre *et al*. 1994
Nosema furnacalis	Insects	13	440 to 1,360	10.2	Munderloh *et al*. 1990
Nosema pyrausta	Insects	13	440 to 1,390	10.6	Munderloh *et al*. 1990
Vavraia oncoperae	Insects	14	130 to 1,930	8	Malone and McIvor 1993
Spragea lophii	Fish	15	266 to 1,076	7.3	Amigó *et al*. 2002
Spragea lophii	Fish	15	271 to 1,120	7.3	Amigó *et al*. 2002
Glugea stephani	Fish	15	340 to 2,654	16.8	Amigó *et al*. 2002
Vavraia oncoperae	Insects	16	140 to 1,830	10.2	Malone and McIvor 1993
Glugea atherinae	Fish	16	420 to 2,700	19.5	Biderre *et al*. 1994
Nosema locustae	Insects	18	139 to 651	5.4	Street 1994
Nosema bombycis	Insects	18	380 to 1,500	15.3	Kawakami *et al*. 1994

By using Condition 3, a difference in band size was detected for samples NC1 and NC2. Band X from sample NC1 appeared to be approximately 81 Kb larger than the corresponding band in sample NC2 (Table 2, Fig. 1C). Condition 3 was run twice on the spore samples and in both runs band X showed a difference in band migration. Bands V and VI in Fig. 1B also show a slight difference in migration pattern between samples NC1 and NC2. The differences in migration pattern were reflected in the mean DNA band sizes for NC1 and NC2 (Table 2).

Genome plasticity and intraspecies variation in karyotype has been documented in microsporidians. Genome analysis of three different strains of *E. cuniculi* gave six different electrophoretic band patterns (Biderre *et al.* 1998). *Vavria oncoperae*, isolated from two different host species (the porina caterpillar *Wiseana* spp. and the grass grub *Costelytra zealandica*), gave karyotype values of 14 and 16 respectively (Table 3). *Spragea lophii* isolates gave karyotypes of 12 and 15 and a genome size of 6.2 Mb and 7.3 Mb respectively (Table 3). Intraspecies variation in electrophoretic band patterns has also been detected among fungi, and protists other than microsporidians (Monaco 1995, Venegas *et al.* 1997). For example, the protist *Leishmania* displays variation in band patterns due to chromosomal rearrangement; the protist *Trypanosoma cruzi* gives different band patterns among isolates obtained from separate geographic areas; and the yeast *Candida albicans* exhibits variation in chromosome number and size which may vary among individual hosts (Malone and McIvor 1993, Monaco 1995, Venegas *et al.* 1997).

The mechanism for inducing variations in chromosome number or size among microsporidians is not well understood. Malone and McIvor (1993) suggested that variations in DNA band patterns might be common among microorganisms with high reproductive rates. The possibility of unequal crossing over during mitosis may account for the variability in chromosome size. Since sexual reproduction can occur in microsporidians and other protists, then there is also the possibility of unequal crossing over in meiosis. Subtelomeric deletions or insertions of repeat sequences may also account for the variability in chromosome size (Monaco 1995, Biderre *et al.* 1998).

The temporal stability of *N. canceri's* karyotype and chromosome size is also an intriguing question. If *N. canceri* exhibits significant plasticity in its genome, then the band pattern should change over time. In a study by Biderre *et al.* (1998) clonal cultures of *T. cruzi* exhibited variations in electrophoretic band pattern over a 5-year period. In contrast, cultures of *E. cuniculi* failed to show any significant variation within the same 5-year period (Biderre *et al.* 1998).

The estimation of *N. canceri's* genome size and karyotype serves as a platform to further understand the molecular biology of the parasite. Our study has shown *N. canceri* spore isolates (obtained from two separate crab species) to have a karyotype of 10 chromosome-sized DNA bands. The size range of *N. canceri's* DNA bands ranges from 280-1,879 Kb for NC1, and 290-1,798 Kb for NC2 (Table 3). The total genome size for *N. canceri* is estimated to be 7.3-7.4 Mb. *N. canceri* is the first crustacean-infecting microsporidian to have its genome characterized, and it will be interesting to see whether other crustacean-infecting microsporidians have a similar karyotype, chromosome size range, and genome size.

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