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Spraguea lophii (Microsporidia) parasite of the teleost fish, *Lophius piscatorius* from Tunisian coasts: Evidence for an extensive chromosome length polymorphism

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ABSTRACT

A microsporidian of the genus *Spraguea* was found parasitizing the nervous tissues of *Lophius piscatorius* collected from various localities in the Mediterranean coastal areas of Tunisia. The tissue localization, the infection focus aspect and sporal dimorphism are characteristics of *Spraguea lophii* species. Molecular data based on partial sequence of SSUrRNA encoding gene shows few nucleotide polymorphisms, compared to all described *Spraguea* isolates. Molecular karyotype obtained on pulsed field gel electrophoresis (1D-PFGE) shows a profile with 14 stained bands in the range of 230–880 kbp and a genome size estimated to 6.700 kbp. The rare cutter endonuclease *Mlu*I KARD 2-D-PFGE fingerprint shows an extensive chromosome length polymorphism, but the number of chromosome is unchanged and consists of 15 different molecules. The extensive chromosome length polymorphism is associated to a reduced number of genetic events.

1. Introduction

The microsporidian *Spraguea lophii* Sprague and Vavra, 1976, is the type species of the genus *Spraguea* in which parasites of several fish species belong to the genus *Lophius*. It was reported for the first time by Thelohan [1] in the spinal ganglia of *Lophius piscatorius* (Linnaeus, 1758) from the Atlantic coast of France. Later, Dolfein [2] reported the same parasite in *L. piscatorius* sampled from Italy and he described it as *Glugea lophii* Dolfein, 1898. The cysts induced by this parasite can reach 2 cm in diameter and have, generally, an aspect of bunch-of-grapes on the level of ganglion cells of the fish nervous tissue. Pace [3] and Weissenberg [4] have proposed to change the name of this Microsporidia to *Nosema lophii* because they didn't observe the sporonte stage, since the schizontes evolve directly to sporoblastes. Weissenberg [5] reported that the parasite forms colonies in the host cell where the infection starts. The cyst contain two regions with two different types of spores 1) an external dark region with oval spores and 2) an internal region with cylindrical spores. Only oval spores are present in newly formed cysts whereas cylindrical spores dominate in old ones. Moreover, Weissenberg proposed that oval spores evolve to cylindrical shapes in old cysts. Sprague and Vavra in a footnote to Weissenberg's chapter suggested changing the name of this parasite to a new genus "*Spraguea*" with a type species *Spraguea lophii*.

A fine ultrastructural characterization of this Microsporidia was carried out, for the first time, by Loubes et al. [6] in examining cysts from the Atlantic Anglerfish, *Lophius budegassa*. This study led to the description of the coexistence within the same cyst of two different forms: a uninucleated form with oval spores localized at the periphery and a binucleated form, recognized by the cylindrical spores with internal localization. Although, no structural or molecular proof was brought, the theory of a unicaryotic symbiosis between two species, one unikaryotic and the other diplokaryotic, was abandoned in favor of the assumption of a single dimorphic species. Takovarian and Cali [7] reported that in the American anglerfish *Lophius americanus* the presence of a microsporidian parasite was forming a *Spraguea* cyst-like infection. The latter revealed that only the uninucleated forms are observed, and thus they proposed to name this parasite *Glugea americanus*.

However, the molecular analysis based on the small subunit rDNA sequence demonstrates the membership of this parasite to the genus *Spraguea* [8]. In fact, Freeman et al. [9] have performed the first molecular phylogenetic analysis of the *Spraguea* as they were describing an infection in the Japanese anglerfish *Lophius litulon*. The parasite was presenting the uninucleated spores only as reported for *L. americanus*. The phylogenetic analysis was based on partial sequences of the SSUrRNA encoding gene. It supports the membership of this

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Microsporidia to the genus *Spraguea* despite the absence of spore dimorphism. Thus, the authors proposed to create a new species, *Spraguea americana*, for the Japan and American isolates [9]. Very recently, a new species with monomorphic spores, *Spraguea gastrophysus* was reported in *Lophius gastrophysus* from the Brazilian Atlantic coasts [10]. Although this species exhibits main phenotypic features of the *Spraguea* genus, the molecular data based on SSUrDNA sequences show a slight divergence with the other species previously reported.

Recently, a fine characterization of the molecular karyotype of the Atlantic isolate of *S. lophii* was carried out by pulsed field gel electrophoresis [11]. The karyotype of *S. lophii* is composed of 15 different chromosomes and presents a haploid state. In the present study we propose an integral approach based on structural, ultrastructural, molecular and genomic methods to give the relative taxonomic position of *Spraguea* isolates from the Northeastern Tunisian coasts.

2. Material and methods

2.1. Host and parasite sampling

Twenty four specimens of *L. piscatorius* were bought from fishermen working on the Northeastern coast of Tunisia (area of Bizerte, 37° 16.4′N, 9° 53. 2′E). The fish were examined in a fresh state for the detection of Microsporidia. Cysts, from the nerves near the kidneys and inside the vertebral column, were collected and then fixed for microscopy analysis or preserved in a phosphate buffer saline 1× solution (PBS) added with penicillin/streptomycin to preserve samples for DNA analysis.

2.2. Light and electron microscopy

For observations of fresh smears, a xenoma is crushed between a slide and a coverslip. Fluorescent observations were made after fixation of a crushed xenoma with methanol and staining with both UVITEX and SYTOX® Green (molecular probe, Invitrogen). The slides were examined with a Leica microscope equipped with epifluorescence and a digital camera.

For ultrastructural studies, the material was fixed with 2.5% glutaraldehyde in cacodylate buffer 0.1 M (pH 7.4) and then post-fixed with 1% osmium tetroxide in the same buffer (v/v) for 1 h. Samples were then washed three times in the same buffer, dehydrated by increasing concentrations of ethanol and embedded in Epoxy resin via propylene oxide. The material was cut with a Leica Ultra-cut S ultramicrotome. Semi-thin sections were stained either with 0.2% Azur Blue II or the fluorescent molecule DAPI (diamidino-2-phenylindole). Ultra-thin sections were stained with uranyl acetate and lead citrate and then observed with a Jeol 1200 EX transmission electron microscope.

2.3. Spore purification and DNA preparation

The purification of *S. lophii* spores and preparation of plugs for pulsed filed gel analysis (PFGE) was performed as described by Mansour et al. [11]. Briefly, 10^8 purified spores were mixed with 100 µl of 1.4% agarose low melting point SeaPlaque_GTG (Cambrex Bio Science, Rockland, ME, USA) cooled to 37 °C in a plug mold. To induce genomic DNA extraction, plugs were incubated with 3 M urea, 0.5 M EDTA for 10 min at room temperature and then treated with 0.5 M EDTA, 0.5% N-laurylsarcosine sodium salt, 2 mg/ml proteinase K (Sigma, Steinhein, Germany) at 55 °C for 48 h.

2.4. PCR and rDNA sequencing

Target DNA was amplified using the PCR primers described by Mansour et al. [12]: forward primer SF4m (5′CACCAGGTTGATYCT GCCTRD3′) and reverse primer SR1147m (5′TGTRGTRAICYTCCGY CAATY3′) or MicR (5′GCGACGGGCGGTGTGTAC3′). Each 50 µl PCR reaction contained 1–10 ng of genomic DNA, 5 pmol of each primer, 0.2 mM of eatch dNTP and 0.5 U of Go Taq® polymerase in 1× supplied buffer with 1.5 mM of MgCl2 (Promega, France). Amplifications were performed on a Perkin-Elmer GeneAmp PCR System. After an initial denaturation at 95 °C for 5 min, samples were subjected to 35 cycles of amplification (denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s and extension at 72 °C for 1 min), followed by 10 min final extension at 72 °C. PCR product was visualized in an Ethidium bromide-stained 1% agarose gel and purified using the QIAquick® PCR purification kit (QIAGEN).

Sequences were determined either directly from the purified PCR products or after insertion into the pGEM®-T Easy Vector system (Promega) and cloning in competent XL1-Blue *Escherichia coli* cells using 5 µl of CEQ Dye terminator Cycle Quick Start Master Mix (Beckman Coulter) in a Perkin Elmer thermocycler apparatus. In addition to the two primers used in the initial PCRs, we have used the SP6 and T7 universal primers for the sequence reaction. Sequencing products were run on a CEQ™ 2000 capillary sequencer (Beckman Coulter). The consensus sequence was based upon both sense and anti-sense strands of samples originating from three positive cloned fragments and four separate PCR products. Assembly of sequences was performed using the CAP3 assembly DNA program (http://pbil.univ-lyon1.fr/cap3.php) [13].

2.5. Sequence analysis and Phylogenetic reconstruction

Sequences were extracted from GenBank according to their BLAST homology score [14]. Consensus sequences were then aligned using CLUSTALX software [15] with default parameters. Phylogenetic tree was conducted using MEGA version 4 [16] with neighbor-joining analyses using the Kimura 2-parameter model. Bootstrap analysis was based on 1000 resamplings [17]. Gene accession numbers of SSUrDNA sequences and relative *Spraguea* hosts are listed in Table 1. Distance estimation was carried out using the Kimura 2-parameter model distance matrix for transitions and transversions.

2.6. KARD 2D-PFGE

PFGE analysis was performed in a Gene Navigator™ system (Pharmacia) in a 0.5× TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). The chromosomal DNA bands were resolved on

Table 1
Selected Microsporidia accession numbers.

Accession number	rDNA sequence coordinates	Parasite	*Spraguea* hosts
AY465876	1-1189	*Spraguea americana* (LL)	*Lophius litulon* (Pac, Japan)
AF056013	1-1199	*Spraguea lophii* (LPB)	*L. piscatorius* and *L. budegassa* (Med, France)
AF056014	1-1010	*Spraguea* (*Glugea*) *americana* (*LA2*)	*L. americanus* (Atlantic, USA)
AF033197	1-1009	*Spraguea lophii* (*LA1*)	*L. americanus* (Atlantic, USA)
AF104086	6-1189	*Spraguea lophii* (*LP*)	*L. piscatorius* (Atlantic, USA)
GQ868443	147-1147	*Spraguea gastrophysus*	*L. gastrophysus* (Atlantic, Brazil)
JF927624	6-1199	*Spraguea lophii*	*L. piscatorius* (Med, Tunisia)
AJ252952	6-1188	*Microgemma vivaresi*	
AF364303	1-1194	*Tetramicra brevifilum*	
AY033054	1-1198	*Microgemma caullery*	
AF356223	6-1222	*Ovipleistophora mirandellae*	
AJ252956	6-1206	*Pleistophora typicalis*	
GAU15987	6-1189	*Glugea atherinae*	

1% agarose gels (molecular grade SeaKem® GTG®; Cambrex Bio Science) at 4.5 V/cm^2, 12 °C for 20 h with 22 s pulses, 13 h with 50 s pulses and then 12 h with 85 s pulses. In the same gel, one plug containing chromosomes of the described Atlantic isolate, *S. lophii* [11] was loaded with the chromosomal DNA of *Saccharomyces cerevisae* YPH80 strain (New England Biolabs) and Lambda ladder PFG markers (New England Biolabs). Gel was stained for 30 min with a solution of 0.5 μg/ml ethidium bromide and photographed under UV transilluminator. Images were saved with a Gel Documentation and Analysis System (Herolab).

For 2D-PFGE analysis, a 1 mm slice in width of agarose of the electrophoretic karyotype containing all chromosomal bands was cut under UV. The DNA was digested overnight in gel with 200 U of the rare cutter endonuclease *Mlu*I (Invitrogen) in 1× high-salt buffer (100 mM NaCl, 50 mM Tris-acetate pH 8, 66 mM $MgCl_2$) at 37 °C. Digested DNA was radio-labeled using 5 μCi of ^{32}P-dCTP (Amersham Bioscience) and 5 U of DNA polymerase Klenow fragment (USB, Cleveland, OH, USA) for 3 h at 30 °C within the restriction solution. Radiolabeled fragments were separated in a 1.2% agarose gel (molecular grade agarose Eurobio) at 6.1 V/cm2, 10 °C for 10 h with 3.5 s pulses, 8 h with 7.5 s pulses and 6 h with 15 s pulses. A mid range (15–300 kb) (New England Biolabs) was used as a marker. For visualization of radioactive DNA fragments, gel was dried for 40 min at 60 °C using a gel slab dryer and exposed to a Biomax™ MS film (Kodak) between two intensifying screens at −80 °C for 24 to 72 h.

3. Results

3.1. Infectious focus and spore characterization

All examined anglerfish were infected by the microsporodian parasite. The size of the cysts varies from 2 mm to 23 mm in diameter and are formed as isolated xenoma (having the aspect of grains) or gathered in one, two or three lobes attached to the same nerve (Fig. 1). Lobes are observed only in the vagal nerve close to the kidney. Isolated cysts (xenoma) are often taken throughout the spinal column. Both of the form and localization of infection foci are characteristics of Microsporidia belonging to the genus *Spraguea* as described by Doflein [2] (Fig. 2). The examination of fresh preparation shows the presence of the two cylindrical and oval spores. Sytox Green fluorescent staining revealed that cylindrical shaped spores are binucleated, whereas oval spores are uninucleated (Fig. 3). Moreover, we noticed that in some examined cysts, spores are uniform and of unikaryotic types. Interestingly, the transmission electronic microscope analysis confirms the presence of the two types of spores as observed in epifluorescence (Fig. 4).

Serial semi-thin preparations of many cysts indicate different evolution forms of the infection foci. Some cysts are with only oval spores whereas others are with the two types of spores (Figs. 5, 6). In some xenoma more than one secondary infection foci of 100–400 μm in diameter occur and form a colony of parasites (Fig. 6). We called this infection focus, micrograin. Several cells with nuclei of ordinary size (i.e. not hypertrophied) have been observed inside the cysts. These cells could correspond to phagocyte cells having infiltrated the xenoma (Fig. 7a) which reflects a transformation of xenoma to granuloma structure. The wall of granuloma is formed by fibroblast cells that are generally irrigated with capillary vessels. The thickness of the cyst-wall varies between 20 and 50 μm (Fig. 7b). These observations support the membership of this Microsporidia to *S. lophii* species.

3.2. Ribosomal sequence comparison and phylogenetic analysis

In total, 1199 nucleotide fragment corresponding to an internal part of the SSUrRNA gene was obtained for the Tunisian isolate *S. lophii* (GenBank deposit accession number JF927624). The SSUrRNA gene sequences are highly conserved among *Spraguea* isolates. We identify one highly variable region approximately in the middle of the rRNA (Table 2). Only two gaps seem to be specific to the Tunisian isolates and are positioned at 170 and 1149 (Table 2). The two Mediterranean isolates are clustering together (Fig. 8).

The Tunisian isolate SSUrDNA sequence is presenting fewer differences compared to the Mediterranean or Atlantic isolates respectively parasitizing *L. piscatorius* and *L. budegassa* than with the Pacific ones. The most divergent sequence is that belonging to the newly described *S. gastrophysus* species (12 insertion/deletions or indels and 11 substitutions) (Table 3). The Kimura-2-parameter pairwise distance shows lowest values with isolates from *L. piscatorius* and *L. budegassa* and a percentage of similarity of about 99.6%. The species from *L. americanus* and *L. litulon* shows a percentage of similarity of about 99.38%. The newest species reported in *L. gastrophysus*, presents a percentage of similarity less than 99% and represents an outgroup in *Spraguea* (boostrap value at 85).

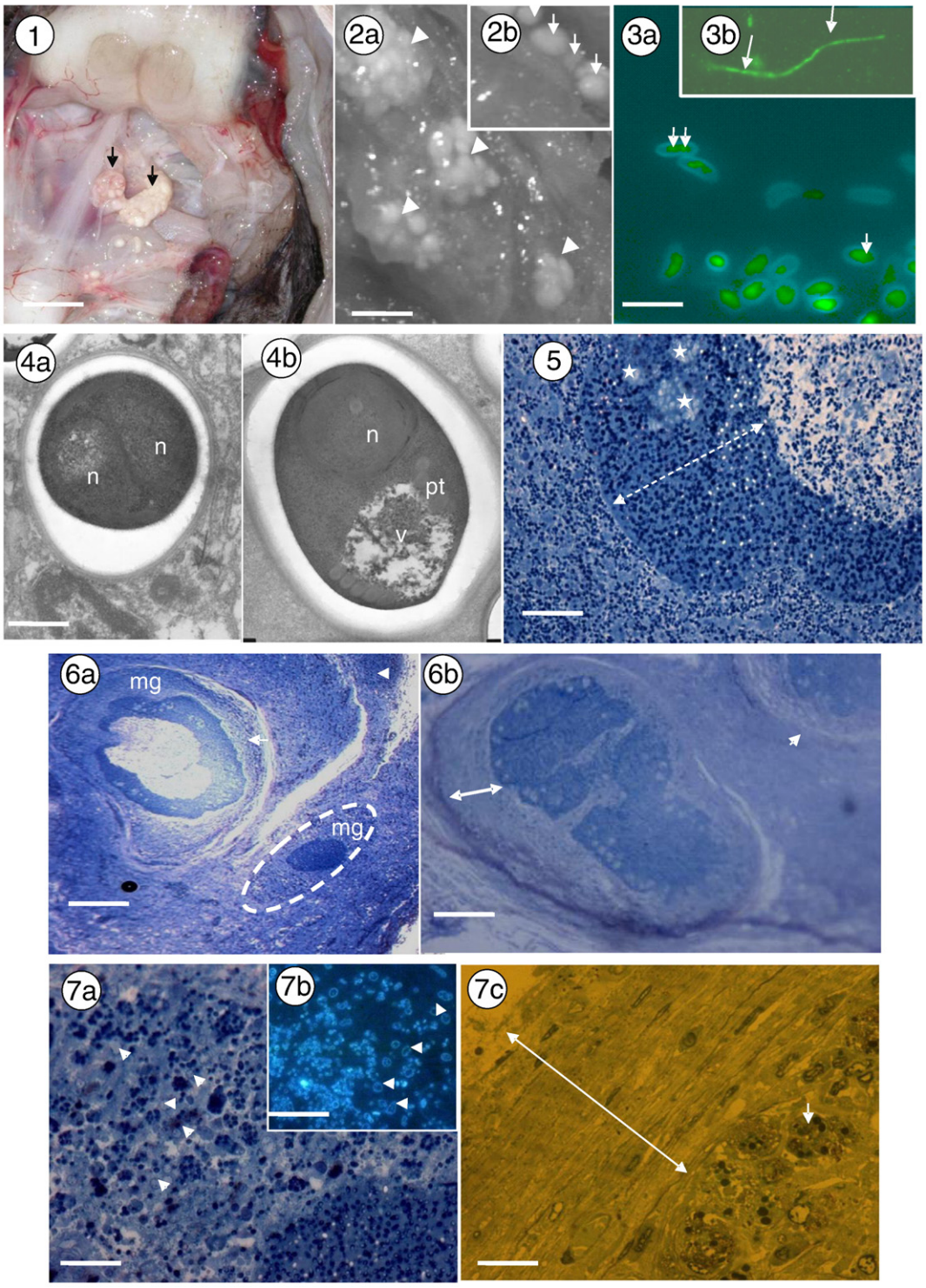
Phylogenetic analysis confirms that *S. lophii* is forming a monophyletic group (Fig. 8).

3.3. KARD 2D-PFGE analysis

The molecular karyotype of the *S. lophii* Tunisian isolate shows 14 bands (Fig. 9) that have a size range between 215 and 880 kbp. There are two bands which are very close to each other at 219 and 223 kbp. We note that the K07 band seems to have a double intensity which suggests a co-migration of more than one DNA molecule. Comparative analysis with the reference Atlantic karyotype shows an important size polymorphism of the karyotype bands. K01 and K07 are the only bands that present no size polymorphism. The distribution of karyotype bands in three different size ranges is a conserved feature of the genome of *S. lophii* (200–300 kbp, 350–500 kbp and 600–900 kbp).

The 2D-PFGE restriction profile was performed with *Mlu*I enzyme to display a more precise genomic fingerprint (Fig. 10). This procedure enables attribution of chromosomes to each karyotype band. Chromosome size is confirmed and refined by the summation of all restriction fragments (Table 4A). As suggested by the 1D-PFGE karyotype description, the K07 band is composed of two chromosomes that present only a 3 kbp size difference (C07 and C08). Chromosome attribution can be performed on the basis of the *Mlu*I restriction profile and accordingly to the description of Mansour et al. [11]. We confirm that the genome of *S. lophii* is composed of 15 chromosomes. It is haploid and has a size of 6.7 Mbp in average (Table 5). The size of the genome of the Mediterranean *S. lophii* isolate is 116 kbp larger than the Atlantic isolate which has been described with the same tools. Chromosomes C08, C09 and C13 have a size difference which is over 40 kbp. The average chromosome length polymorphism (CLP) between the Atlantic and Mediterranean isolates is 25 kbp. Two chromosomes have a conserved size (C01 and C07). The largest chromosome (C01) is the only molecule that remains unchanged in the *S. lophii* genome regarding its size and *Mlu*I restriction pattern. Two chromosomes C09 and C12 are presenting extensive modifications of their *Mlu*I restriction profile. Their identification was possible only because of the genomic context.

The reference KARD (Karyotype and Restriction Display 2D-PFGE) performed on the Atlantic isolate provided 79 *Mlu*I restriction sites while 85 *Mlu*I restriction fragments have been identified in the genome of the Tunisian *S. lophii* isolate. Interestingly, 46/79 fragments of the *Mlu*I restriction sites are conserved between the reference KARD and the present *S. lophii* isolate (gray background in Table 4A and 0 values in Table 4B). The level of conservation between isolate appears to be higher with the 2D-PFGE analysis than it would have been expected from the 1D-PFGE karyotype description. We note that about 60 kbp of additional DNA is associated to new *Mlu*I restriction fragments (labeled n in Table 4B).



Figs. 1–7. *Spraguea lophii* infection in *Lophius piscatorius* showing the organization of infection foci on nervous tissues characterizing this species. Fig. 1: position of the cysts after ventral dissection of the infected host (black arrows). Fig. 2a: gathered cysts (lobes) throughout the spinal column (white arrowhead). Fig. 2b: individual cysts (xenoma) inside the spinal column (white arrows). Fig. 3a: mature spores stained with both UVITEX (blue) and SYTOX Green, showing the monkaryotic (n) oval spores and the diplokaryotic cylindrical spores. Fig. 3b (inset Fig. 3a): polar tube extrusion stained with SYTOX Green (arrows). Fig. 4: transmission electron micrograph sections of mature spores showing the binucleated spores (Fig. 4a) and the uninucleated spore (Fig. 4b). Fig. 5: semi thin section of a xenoma showing the well defined two areas: peripheral area with oval spores and internal dark area (double dashed arrow) supporting the dimorphic character of this species. Fig. 6a: colony of infection focus (micrograin: mg) within a xenoma semi-thin section. Early deposit fibrillar material around the micrograin (arrow). Newly formed micrograins are not delimited. Fig. 6b: a micrograin rounded by a relatively thick fibrillar material forming an envelope (double arrow). In this section we don't observe the two areas inside the infection focus. Fig. 7a: the infection focus is invaded by phagocyte host cells. We clearly observe the nuclei of phagocyte cells by either Blue Azur II or the Dapi. (Fig. 7b). Fig. 7c: the granuloma is limited by a thick envelope formed by fibroblast and irrigated by vessels (double arrow). Phagocyte cells packed with spores are also observed at the periphery (white arrowhead). Scale bars: 1a, 2 cm; 2a, 2b, 8 mm; 3a, 3b 10 μm: 4a, 4b, 0.5 μm; 5, 15 μm; 6a, 35 μm; 6b, 25 μm; 7a, 7b, 15 μm; 7c, 12 μm.

Table 2
Spraguea spp. SSU rDNA sequences comparison and nucleotide mutations location question marks (?) correspond to missing sequences, dashes (−) represent gaps.

Gene accession number	Position																		
	112	170-1	258	376-278	403-407	415-432	500	522	636	720-21	750	774	794	923-24	999	1018	1076	1136-38	1149-1150
JF927624	–	– –	G	GAG	– –CAC	GGGG– – – – – GTATTCGAT	A	A	–	– –	C	G	G	GA	–	A	–	AA–	– –
AF104086	–	TG	G	GAG	–CCAC	–CTG– – – – – –G– G–TC–A	A	–	–	– –	C	G	G	– –	–	–	–	AGG	AT
AY465876	–	TG	G	GAG	ACCAC	ACTG– – – – – –G– GGTC–A	–	A	–	– –	C	G	G	GA	–	A	–	GG–	AT
AF033197	T	TG	G	GAG	ACCAC	ACTGT– – – –GG– GGTC–A	–	A	T	GA	C	G	G	GA	C	A	G	GG–	AT
AF056013	?	??	G	GAG	ACCAC	ACTGGAGGTGA– GTC– –A	–	A	–	– –	G	G	G	GA	–	A	–	AA–	AT
AF056014	?	??	G	GAG	ACC– –	ACTGGAGGTGA– GACT–A	–	A	–	– –	G	G	G	GA	–	A	–	GG–	AT
GQ868443	T	TG	A	A– –	ACCAC	ACTGGAGGT– – – – – –C–A	–	A	–	– –	C	C	A	GA	–	A	–	GG–	AT

4. Discussion

On the basis of extensive surveys, the present study represents the first report of this microsporidian species on the African coasts. The description of the parasite was undertaken following an integral approach at different levels using structural, ultra-structural, genomic and molecular tools. The location and aspect of infection focus are in agreement with a previous description of *Spraguea* isolates [4,6,7,9–11,18,19]. Structural analysis of extracted xenoma confirms the presence of the two shaped spores; cylindrical and oval ones. Ultra-structural study and Uvitex stain revealed that cylindrical spores are binucleated whereas oval spores are uninucleated. According to structural and molecular analyses, the Tunisian isolate is considered to be a member of the *S. lophii* species. In fact, dimorphism is not always observed in the *Spraguea* species. Takovarian and Cali [7] reported that the American anglerfish, *L. americanus*, could be infected by a *Spraguea*-like Microsporidia forming only uninucleate spores. A similar Microsporidia forming only uninucleated spores were also reported by Freeman et al. [9] in a Japanese anglerfish *L. litulon*. Molecular analysis confirms the membership of these uninucleated parasites to the genus *Spraguea* [8–10,20,21]. The American and Japan isolates that have been proposed to form a new species, *S. americana* [9]. More recently Casal et al. [10] reported a new *Spraguea* species parasitizing *L. gastrophysus* from the Brazilian Atlantic coasts. This species is also characterized by the presence of monomorphic ellipsoidal spores. The membership of this parasite to the genus *Spraguea* was confirmed by molecular analysis based on SSUrDNA sequences even though it represents an outgroup in the *Spraguea* phylogeny (Fig. 8). The Tunisian isolate belongs to the *S. lophii* cluster and presents a higher percentage of identity with the other Mediterranean sequence isolate in France. The level of polymorphism does not exceed 1% between the different sequences *S. lophii* and *S. americana*. Only the Brazilian isolate shows 1.05% of nucleotides differences. Therefore, we conclude that *S. lophii* and *S. americana* are forming a monophyletic group of Microsporidia infecting anglerfish.

Further studies will have to confirm the close relationship found between the two Mediterranean isolates. In fact, the Mediterranean Sea connects through the Strait of Gibraltar to the Atlantic Ocean in the west. This strait is too small to support intensive fish population mobility. We could expect that significant differentiation among geographical groups of the Atlantic and the Mediterranean anglerfish populations have begun to occur which should also be the case for

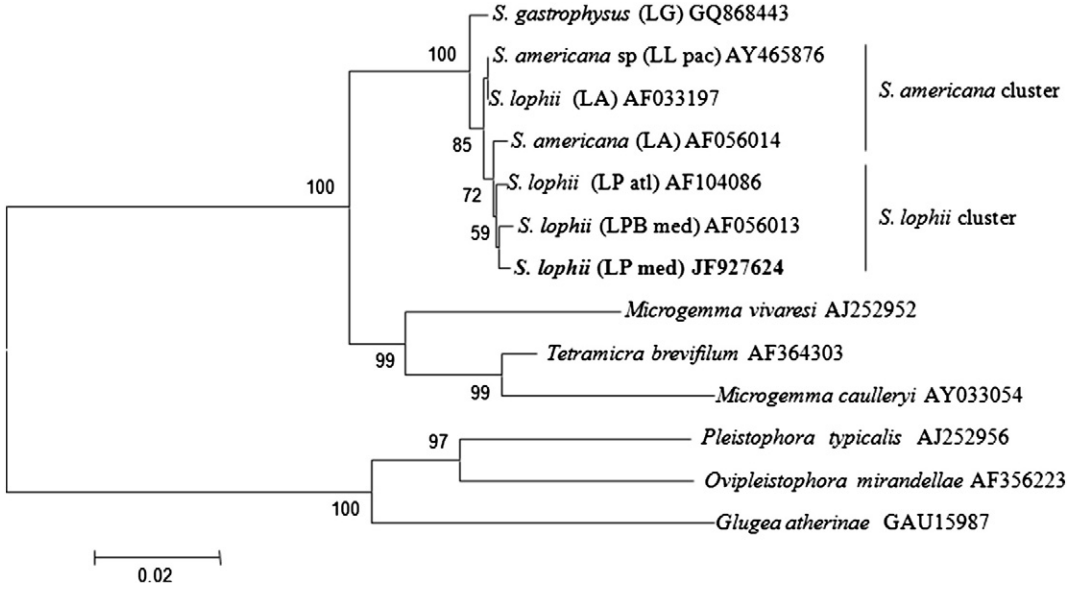


Fig. 8. Phylogenetic tree of *Spraguea lophii* for different submitted sequences and related species based on the SSU rDNA partial sequence using Neighbor joining method. *Glugea atherinae*, *Pleistophora typicalis* and *Ovipleistophora mirandella* are used as outgroups. Numbers in branches represent bootstrap percentages of 1000 replicates. The GenBank accession number for each sequence is given adjacent to the corresponding species. The scale bar indicates the number of changes per site. Tunisian isolate is indicated in bold. For *Spraguea* species, fish hosts are noted in uppercase in parentheses: LP: *Lophius pisctorius*, LB: *Lophius budegassa*, LPB: *L. pisctorius* and *L. budegassa*, LA: *Lophius americanus*, LL: *Lophius litulon*, LG: *Lophius gastrophysus*. atl: Atlantic ocean isolate, med: Mediterranean sea isolates, pac Pacific Ocean isolate.

Table 3
Genetic distance estimation using the Kimura 2-parameter, obtained after pairwise analysis between different *Spraguea* isolates and the Tunisian one. Number of indels and substitutions are reported.

Spraguea sequence accession GenBank number (hosts)	Pairwise distance (Kimura 2-parameter)/percentage identity	Indels (gap/ins)	Substitution (transition/ transversion)
AF056013 (LPB)	0.0041/99.59	10 (9/1)	1 (0/1)
AF104086(LP)	0.0041/99.59	10 (5/5)	1 (1/0)
AF033197 (LA1)	0.0062/99.38	15 (14/1)	2 (2/0)
AF056014 (LA2)	0.0063/99.37	11 (9/2)	4 (3/1)
AY465876 (LL)	0.0062/99.38	7 (6/1)	2 (2/0)
GQ868443 (LG)	0.0105/98.95	12 (6/6)	11 (5/6)

their parasites. In our study, we clearly show that the genome of *S. lophii* is haploid and the two nuclei contain the same genome. The first description of the molecular karyotype of a *Spraguea* genome has been reported by Biderre et al. on an Atlantic isolate [22]. Later and still on an Atlantic isolate, a relationship between karyotype band and chromosome number based on a *Mlu*I and a *Sma*I restriction pattern has been described by Mansour et al. [11]. Amigo et al. [23] have first reported a karyotype comparative analysis between a Mediterranean and an Atlantic *Spraguea* isolate. They established that the genome of *S. lophii* is composed of 15 DNA molecules. Many chromosome length polymorphisms (CLPs) were observed between the two isolates. The haploïd nature of the *Spraguea* genome was definitively stated by 2D-PFGE KARD analysis [12]. Ploidy status of various Microsporidia genomes was established by this approach (*Encephalitozoon cuniculi* [24–26], *Brachiola algerae* [27] and *Paranosema grylli* [28]). The Tunisian *S. lophii* isolate exhibits 14 well resolved karyotype bands whereas only 13 bands were described in the Atlantic isolate (Fig. 9). Chromosome Length Polymorphisms (CLP) concerns most of the molecules (13/15 bands) and they represent more than 10% of the size of the molecule. Many CLPs are associated to several modifications of the *Mlu*I restriction pattern suggesting that more than one genetic event may be involved. This observation improves the idea that 1D-karyotype profiles do not provide relevant information to compared close isolates.

The 2D-PFGE analysis offers a higher resolution of the genome. At this scale, the polymorphism concerns 40% of the restriction fragments. Interestingly, they are associated to a reduced number of genetic events. For example, chromosome C02, presents 6 differences between the Tunisian Mediterranean isolate and the Atlantic isolate described by Mansour et al. [11]. It corresponds to only 4 genetic events: 2 insertions and 2 fusions (Tables 4A and 4B). This is much more interesting for further comparison of populations that may have been separated for million years. The aim of the present discussion is to evaluate the different genetic events and to discuss how they can be related to a time scale. Six genetic events are associated to the gain or loss of restriction sites. This is in agreement with the slow evolution of the DNA sequence. The coding density is expected to be around 90% as in many other small eukaryotic genomes. Most *Mlu*I restriction sites are associated to coding sequences but the *Mlu*I sites associated to non-coding region might be more susceptible to accumulate point mutations. In two cases (C04 and C12), the gain/loss of *Mlu*I sites is associated to a larger rearrangement of the locus.

The most frequent polymorphisms are indels. They are rarely more than two events per chromosome. Chromosome Length Polymorphisms (CLP) associated to unequal recombination at chromosome end have

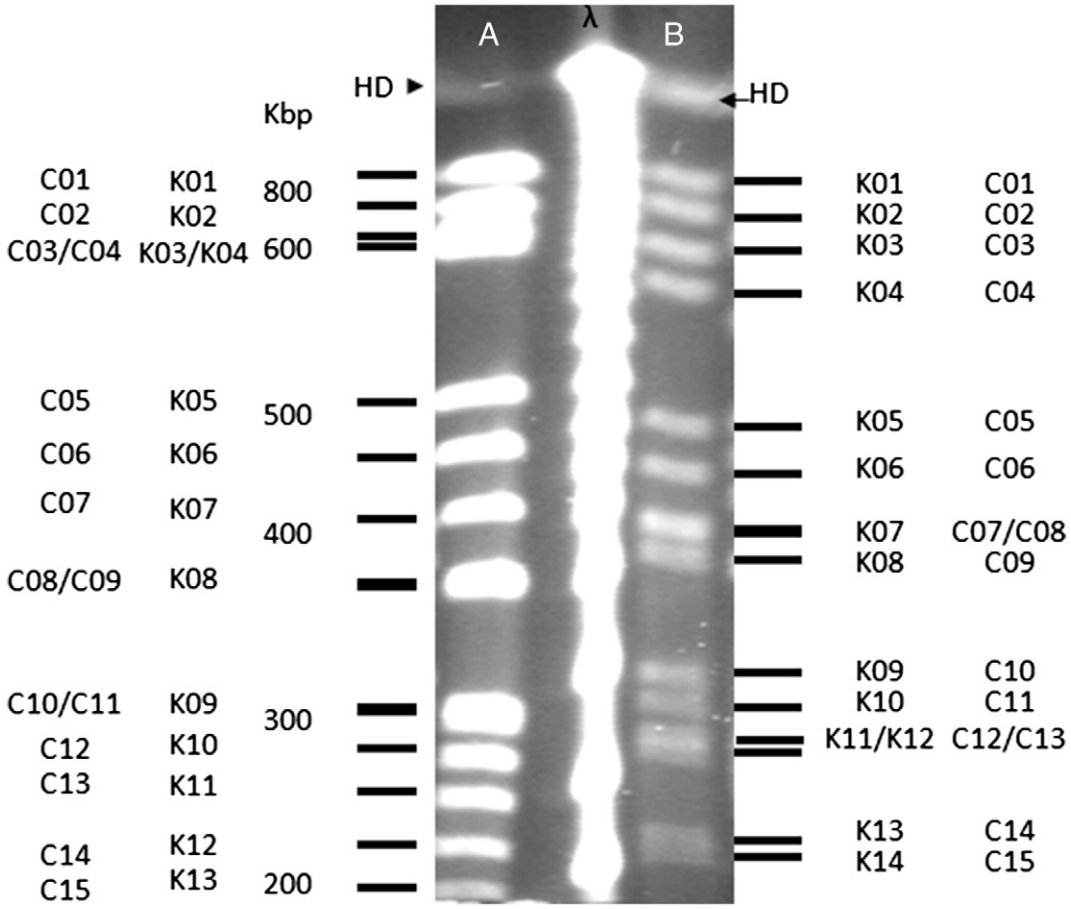


Fig. 9. Molecular karyotype of *Spraguea lophii*: A) the Atlantic isolate loaded as reference. B) The Mediterranean isolate resolved in the same condition as the Atlantic one. Approximately DNA sizes were estimated using a Lambda (λ) DNA marker and the Atlantic isolate. The correspondence between band (K01 to K14) and chromosome molecule (C01–C15) is given after KARD 2D-PFGE analysis.

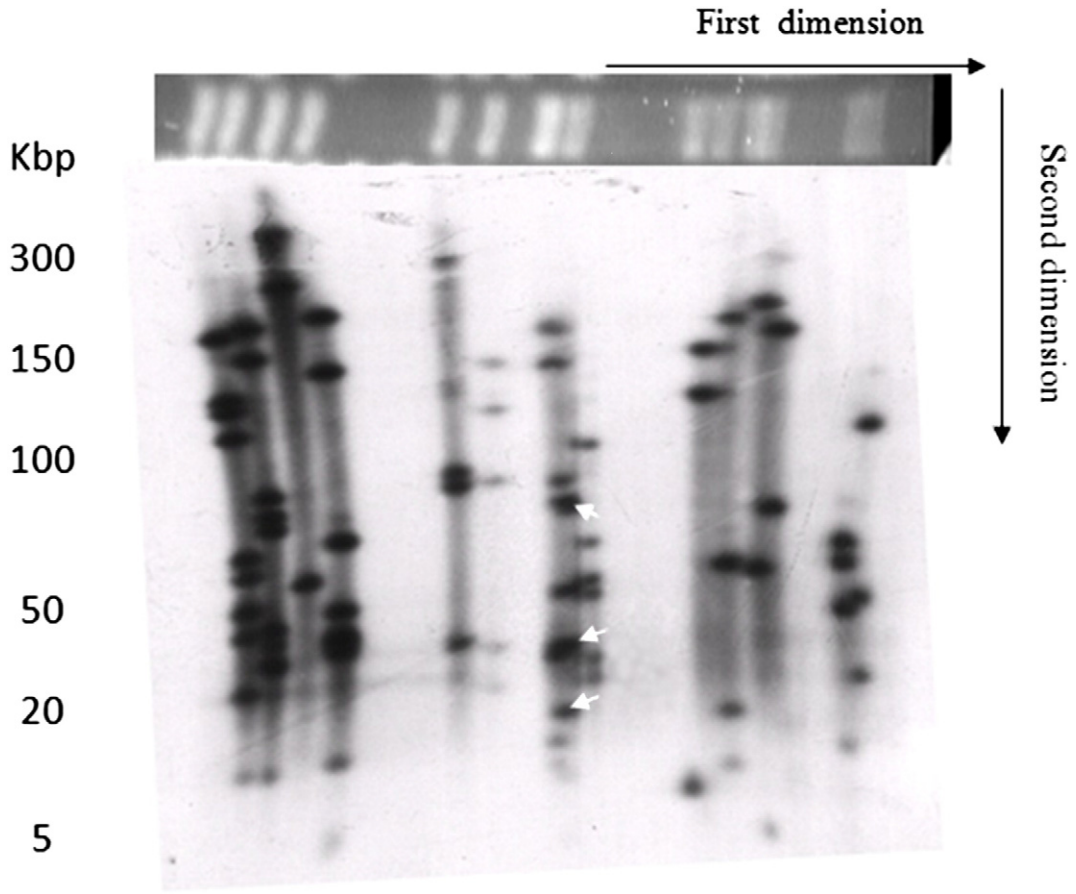


Fig. 10. Restriction *Mlu*I KARD 2D-PFGE profile of the whole genome of the Tunisian isolate *Spraguea lophii*. We observe a slight shift between C07 and C09 restriction fragments (arrows).

been described for a large number of protozoan and some human and animal parasite such as *Plasmodium* spp., *Trypanosoma* spp., *Leishmania* spp., *Giardia lamblia*, *E. cuniculi* and *Pneumocystis carinii* [26,29,30–36]. The CLP were considered as a genetic marker of the recombination that takes place in gene families encoding surface protein which is part of the immune evasion strategy developed at genetic level in parasites [36,37]. Indeed, chromosome end often carries gene that encodes surface antigens that are part of multigenic families [38–43]. Considering the resolution of our analysis; the recombination events at chromosome ends should take place more often between the telomere and the most distal *Mlu*I restriction site. We consider that most of the RFLP observed are related to chromosome end rearrangements. Hybridization with a telomeric probe would confirm this hypothesis.

A fine analysis revealed that some indels may correspond to different types of recombination events. First, we observed that chromosome C07 and C08 are presenting an insertion and a deletion of the same size. It suggests that these two polymorphisms might be related to single inversion events overlapping the restriction site. These events are expected to be rare and might be associated to a speciation process. The second feature is that half of the indels are presenting a size of about 10 kbp. It is also the average size of the new fragments on chromosomes C10, C11 and C13. We may suspect that some of these RFLP correspond to transposition events. In fact, the presence of this type of elements in *S. lophii* was suggested by a random sequencing of the genome [44]. LTR associated transposable elements have been described in *Nosema bombycis* [45,46]. LINE and SINE elements were also

Table 4A
Distribution of *Mlu*I restriction fragment size (kpb) of *Spraguea lophii* genome. Sizes were estimated on the basis size standard neighbor spots and 2-DPFGE KARD of reference isolate. The size of each molecular chromosome (C01–C015) is estimated according to the first dimension and the summation of restriction fragments generated by *Mlu*I.

A. *Mlu*I restriction site analysis (size, sum and numbers).

C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	C13	C14	C15
180	185	360	205	260	150	175	125	160	160	182	187	176	77	128
145	160	255	155	102	141	85	77	98	145	70	70	85	72	55
140	88	63	80	99	99	55	58	90	12	20	10		54	32
130	77		55	39	40	40	54	37	12	20				
73	62		48		32	22	37	15		13			15	
60	49		46			22	35	13						
53	42		15			12								
46	40		10											
30	35													
16	35													
5	15													
878	788	678	614	498	452	411	386	413	329	305	267	261	218	215

Table 4B
Size difference in kbp between the Tunisian and Atlantic *S. lophii* KARD (Karyotype and restriction display 2D-PFGE).

C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	C13	C14	C15
0	0	0	−115	−20	0	0	+17	+65	0	−5	+5	0	0	+13
0	0	0	+10	0	+10	0	0	+18	+10	+15	70^	+20	+10	+5
0	+10	−12	80i	0	0	−18	+23	−3	12i	0	10^		0	−5
0	+5	10d	55i	0	−15	+3	−23	0	12i	20^			−10	
0	0		0		0	−3	0	−18		13^				
0	0		0			0	0	−15						
0	42*		0			12i								
0	0		0											
0	0		46d											
0	0		37d											
0	0													
0	+15	−22	−63	−20	−5	−6	+17	+47	+34	+10	+5	+20	−2	+13

+: the fragment is larger than in the reference karyotype of *S. lophii* from the Atlantic Ocean.
−: the fragment is smaller than in the reference karyotype of *S. lophii* from the Atlantic Ocean.
*: fragment resulting from the fusion of two *Mlu*I restriction fragments (RFLP).
^: fragment resulting from the splitting of a *Mlu*I restriction fragment (RFLP).
i: additional fragment (rearrangement with insertion).
d: deleted fragments (rearrangement with deletion).

found at the extremity of a megabase chromosome in *Glugea atherinae*, a fish microsporidian parasite (E. Cornillot, personal communication).

To summarize the fine karyotype analysis, we can say that the two genomes could be first distinguished by their chromosome C09. Then, we observe that less than 10% of the *Mlu*I restriction sites are not conserved and this is in agreement with the level of coding region in the genome. Four site loss or appearances are associated to large rearrangements. Insertion/deletion events are associated to internal dynamics of the *S. lophii* genome. This fine analysis of the karyotype explains why the 1D-karyotype profile is not sufficient to differentiate parasite population. The *Mlu*I based RFLP analysis in 2D-PFGE enables us to characterize specific events that may help to differentiate the Atlantic *S. lophii* population from the Mediterranean parasite population. Interestingly, the number of variable sites is higher than in the SSUrRNA gene sequence and lower than in the karyotype. This property could later be used to analyze the parasite population of a single area and to differentiate it from other areas.

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Table 5
Comparative analysis of S. *lophii* PFGE genome. Data supports the existence of CLP. All works support the existence of 15 chromosomes. Note that chromosome number has been estimated by digital processing of scanned gel for Amigo et al. works.

Band number	*S. lophii* (LP) Atl		*S. lophii* (LP) Atl		*S. lophii* (LB) Med		*S. lophii* (LB) Atl		*S. lophii* (LP) Med	
	Sizes kb (kbp)	CDM	Sizes kb	CDM	Sizes kb	CDM	Sizes kb	CDM	Sizes kb	CDM
K1	980	Unknown	1076	1	878	1	1120	1	878	1
K2	920		887	1	788	1	915	1	775	1
K3	750	Unknown	767	1	678	1	796	1	700	2
K4	700	Unknown	679	1	614	1	607	1	520	1
K5	540	Unknown	490	1	490	1	510	1	467	1
K6	480	Unknown	440	1	452	1	453	1	417	1
K7	400	Unknown	388	1	413	2	423	2	369	1
K8	350	Unknown	353	2	386	1	374	1	295	2
K9	310	Unknown	301	3	329	1	314	4	262	1
K10	300	Unknown	266	1	305	1	295	2	241	1
K11	260	Unknown	271	2	267	1			218	1
K12	230	Unknown			261	1			202	1
K13		Unknown			218	1				
K14					215	1				
Number of bands/DNA molecules	12	?	11	15	14	15	10	15	13	15
Estimated genome size (Mbp)	6220		7272		7287		6672		6710	
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KB: karyotype bands; CDM: chromosomal DNA molecule; Atl: Atlantic; Med: Mediterranean.

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