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# Biology of a new xenoma-forming gonadotropic microsporidium in the invasive blotchfin dragonet *Callionymus filamentosus*

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ABSTRACT: A gonadotropic microsporidian parasite, *Obruspora papernae* gen. et sp. nov. (Microsporidia: Enterocytozoonidae), is described from *Callionymus filamentosus* (Teleostei: Callionymidae) in the Mediterranean Sea. The host, a Red Sea invasive species which entered the Mediterranean through the Suez Canal, was first collected in the Levant Basin in 1953, whereas its parasite went unobserved until 2008. Analysis of partial small subunit ribosomal gene sequences (SSU rDNA) placed the new species within the *Nucleospora, Desmozoon*, and *Paranucleospora* clade, and as it differs from each of them, it is assigned to a new genus. The development of the parasite is described, and the biological mechanisms underlying this parasite–host system are analyzed. Prevalence of infection approached 80% in female samples throughout most of the year. Males showed no signs of infection, but parasite rDNA was detected in male internal organs. The parasite-induced xenomas progressively occupied and eventually replaced much of the ovary, in some cases producing effective castration. Despite high levels of parasite infection, current trawl fishery statistics indicate that the abundance of Mediterranean populations of the host remains high. The parasite impact on the host population dynamics is unclear. Possible effects of the new microsporidian parasite on the reproductive effort of *C. filamentosus* and the potential role of another parasite, the ectoparasitic copepod *Lernanthropus callionymicola*, as an additional host in the life cycle of *O. papernae*, require further investigation.

KEY WORDS: Invasion · Red Sea · Suez Canal · Mediterranean Sea · *Callionymus filamentosus* · *Lernanthropus callionymicola* · *Obruspora papernae*



## INTRODUCTION

The blotchfin dragonet *Callionymus filamentosus* Valenciennes, 1837, is a small, demersal fish widely distributed in the Indian Ocean (Fricke 2001). Via the Suez Canal, this species has invaded the eastern Mediterranean, where it was first documented at depths of 20 to 100 m by Ben Tuvia (1953). It has since spread along the Levantine coastline (Golani et al. 2002, Corsini-Foka 2010) and is regularly reported from trawl landings in the region (Golani & Bernardi 2012). In Israel, it is the most abundant species found in commercial trawl nets at depths of 20 to 40 m (Stern 2010).

Microsporidia are obligate, eukaryotic, intracellular spore-forming parasites considered to be highly reduced Fungi (Lee et al. 2008). They parasitize a wide range of hosts, tissue, and cell types. Sixteen

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genera comprising approximately 120 species have been described from fish (Kent et al. in press). Microsporidium development is simple and typically includes merogonial and sporogonial stages which in most cases are transmitted directly between host individuals (Lom & Nilsen 2003), although the life cycles of some species have been shown to involve both crustaceans and fish (Nylund et al. 2010). The field data on microsporidian infections in fish populations in the Mediterranean are extremely sparse, and only 1 study has documented an infection in a population of wild stingray *Dasyatis pastinaca* (Linnaeus, 1758) (Diamant et al. 2010).

In 2008, microsporidian xenomas were noted on the ovaries of *Callionymus filamentosus* collected at Antalya Bay, Turkey, and later that year off Ashdod, Israel. *C. filamentosus* is also infected by the ectoparasitic gill copepod *Lernanthropus callionymicola* (El-Rashidy & Boxshall 2012). Ectoparasitic copepods have been shown to be involved in the life cycle of microsporidia that infect their fish hosts (Freeman & Sommerville 2009, Nylund et al. 2010), and the potential role of *L. callionymicola* as a vector in the transmission and development of the *C. filamentosus* microsporidium warrants consideration.

## MATERIALS AND METHODS

*Callionymus filamentosus* were collected throughout 2008 to 2011 in the Mediterranean coastal waters of Israel and Turkey; additional samples were collected in Eilat, Gulf of Aqaba, at the northern end of the Red Sea. Specimens were examined externally and internally for parasites and lesions. Representative samples of tissue were examined fresh or fixed and preserved for subsequent processing.

### Sample collection

Specimens of *Callionymus filamentosus* on the Israeli Mediterranean coast were collected by otter trawl (40 mm mesh size at cod end), at 20 to 40 m depth, at 2.8 knots, for 90 min. They were then examined externally, weighed, measured, and dissected. In the Red Sea, fish were collected by beach seine and similarly processed. Further samples were obtained from commercial fishermen working off the Israeli Mediterranean coast, and an additional 151 preserved specimens collected between 1974 and 2004 from the fish collections of the Steinhardt National Natural History Museum and Research Center, Tel Aviv University, and the Hebrew University Zoological Museum, Jerusalem, were examined.

Upon dissection, intensity of infection in females was determined on a 4-point scale: (1) low intensity with few small, isolated xenomas, (2) intermediate intensity with a large number of xenoma patches, (3) highly infected ovaries with some visible ovarian tissue, and (4) heavily infected ovaries with little or no ovarian tissue.

### Light microscopy

Fresh tissue imprints of the microsporidian xenomas were taken on glass slides, air-dried, fixed in absolute methyl alcohol, and stained with Giemsa or Gram stains. Pieces of normal and infected gonad tissue were fixed in 10% buffered neutral formalin for 48 h, rinsed, and stored in 70% ethanol, for subsequent paraffin histology. Blocks were prepared according to routine histology procedures at the National Center for Mariculture, Israel (NCM) (Diamant et al. 2010). Blocks were sectioned on a Leica microtome, and 7 µm sections were stained with hematoxylin and eosin (H&E). Micrographs were taken with an Olympus 4040 camera mounted on an Olympus BX-40 microscope.

### Transmission electron microscopy (TEM)

Ovarian tissue samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed with osmium tetroxide, stained en-bloc with uranyl acetate, and embedded in Agar-100 resin blocks. Thin sections were cut with a diamond knife on an LKB ultratome III, stained with lead citrate, and mounted on copper grids. The grids were examined under a Jeol 100CXII transmission electron microscope at 60 KV.

### Extraction and amplification of microsporidian DNA

Gonad tissue containing a xenoma from a *Callionymus filamentosus* female caught in November 2009 at Ashdod (Israel) was preserved in 70% ethanol. Lysis was performed using 1 ml of TNES buffer (Asahida et al. 1996) and 40 µl of Proteinase K (20 mg $ml^{-1}$). DNA was extracted by a standard phenol–

chloroform protocol, followed by ethanol–sodium acetate precipitation.

A 1636 bp rDNA sequence including most of the 16S rRNA, the ITS-1, and the 5′ end of the 23S rRNA was amplified using the primers MIC-F-New and 580r (Table 1). The obtained amount of DNA was insufficient for direct sequencing, so nested PCR amplifications were conducted using the primer pairs MIC-F-New/Mic-1321-R-New (1140 bp) and New-530f/580r (1250 bp). The 2 overlapping fragments obtained were directly sequenced on both strands with an ABI PRISM 3100 (Applied Biosystems) genetic analyzer. An identical sequence was also obtained from the infected gonads of a different fish collected at the same time (data not shown). The microsporidium sequence was deposited in the EMBL-EBI European Nucleotide Archive (ENA) under accession number HG005137.

Additional tissue taken from different organs of 13 *Callionymus filamentosus* males was tested for the presence of microsporidian DNA. Tested organs were gills, testes, kidney, heart, and liver. Total DNA of each organ was extracted using 300 µl SDS buffer following Sambrook et al. (1989). The amplification of the 5′ region of the *C. filamentosus* microsporidium small subunit ribosomal DNA (SSU rDNA) was performed using nested PCRs with the primer pairs MIC-F-New/Nucleo-int-R1 and ES1-short/Nucleo-int-R1 for the first and second amplifications, respectively (Table 1).

### Phylogenetic analysis of the microsporidium sequence

Sequences representative of the Enterocytozoonidae diversity were included in our phylogenetic analysis, as well as their closest Terresporidia relatives (cf. Freeman & Sommerville 2009, Tourtip et al. 2009, Nylund et al. 2010, Stentiford et al. 2011, Jones et al. 2012, Freeman et al. 2013). Because the *Callionymus filamentosus* microsporidium was ovarian, we also included sequences of *Ovipleistophora* Pekkarinen, Lom & Nilsen 2002 (Marinosporidia), which exclusively targets fish oocytes (Pekkarinen et al. 2002), and some additional Marinosporidia sequences.

The dataset that included 54 sequences was aligned with the program MAFFT version 6 (Katoh & Toh 2008) using the L-INS-i option. Unreliable columns were removed from the alignment using Guidance (Penn et al. 2010a,b). Specifically, columns that scored over 0.93 and with a gap proportion of $<0.4$ were kept. The final data set included 1060 characters of which 153 were constant and 531 parsimony informative. Pairwise divergence among sequences in the unedited alignment were derived from p-distances calculated using MEGA 5.2 (Tamura et al. 2011), with pairwise deletion of gapped positions.

Maximum likelihood (ML) and Bayesian analyses were conducted. The ML tree reconstruction was

Table 1. Names and sequences of the primers used to sequence the ribosomal DNA (*rDNA*) and cytochrome oxidase (*cox1*) genes

| Gene | Primer | Sequence (5′ – 3′) | Source |
|---|---|---|---|
| *rDNA* | MIC-F-New | CAC CCG GTT GAT TCT GCC TGA CG | Based on the MIC-F primer of Nylund et al. (2010) |
| | 580r | GGT CCG TGT TTC AAG ACG G | Vossbrinck et al. (1993) |
| | New-530f | GTG CCA GCA TCC GCG G | Based on the 530f primer of Vossbrinck et al. (1993) |
| | Mic-1321-RNEW | ATA GTG ACG GGC GGT GTG TAC | Based on the 16S Micro1321 Rev primer of Wolinska et al. (2009) |
| | Nucleo-int-R1 | TAT ACC GTG CTCCCTATCCGCTC | Newly designed based on the sequence of *Obruspora papernae* |
| | ES1-Short | GCT AGC CTC TAA GAT TTA GCC ATG C | Based on the ES1 primer of Gresoviac et al. (2000) |
| *cox1* | LCO-1490 | GGT CAA CAA ATC ATA AAG ATA TTG G | Folmer et al. (1994) |
| | HCO-2198 | TAA ACT TCA GGG TGA CCA AAA AAT CA | Folmer et al. (1994) |
| | Cope-BCpos3-D | TTG TAC TTA ATT AGG GGG GTG TGA TC | Newly designed based on the copepod sequence |

carried out using RAxML 7.4.2 with 100 starting trees. Bootstrap percentages (BP) were computed based on 100 bootstrap replicates. The GTR + Γ model was used, as recommended in the RAxML manual.

The Bayesian analysis was performed under the GTR-CAT model of sequence evolution using the program PhyloBayes 3 (Lartillot et al. 2009). Two independent chains were run for 80 000 cycles (ca. 8 000 000 generations). Chains were sampled every 10 cycles, and the first 20% of the cycles were discarded as burn-in. The corrected maximum difference (*maxdiff*) observed across all bipartitions was 0.025, and the average difference was 0.002, which indicates a good run according to the PhyloBayes manual. The remaining parameters reached an effective sample size greater than 900 and a real difference value smaller than 0.1. Branch support was assessed by posterior probabilities (PP). The sequence alignment and trees are available in the TreeBASE repository, http://treebase.org/treebase-web/search/study/summary.html?id=15207.

### Copepods

*Lernanthropus callionymicola* from the gills of *Callionymus filamentosus* males and females (with or without xenomas) were preserved in 70% ethanol. Total DNA was extracted from 34 copepods from Ashdod (Israel) and Iskenderun (Turkey) using either the DNeasy® Blood & Tissue Kit (Qiagen) following the supplementary protocol for purification of total DNA from ticks for detection of *Borrelia* DNA (www.qiagen.com/literature/render.aspx?id=530), or using the SDS protocol described above for fish organs.

The DNA extracts from copepods were used to amplify 2 fragments. An attempt was made to amplify the 5′ region of the microsporidium sequence from *Callionymus filamentosus* ovary in order to determine whether the copepods are a potential vector of the microsporidium. Amplification was performed with nested PCRs as previously described for fish organs. In addition, the barcoding marker was amplified for several samples using nested PCRs with the primer pairs LCO-1490/HCO-2198 (Folmer et. al. 1994) and Cope-BC-pos3-D/HCO-2198 for the first and second amplifications, respectively (Table 1). The same barcoding sequence was obtained for 2 non-infected copepods and 2 copepods that appeared to contain the microsporidium sequence. The barcoding sequence of the copepods was submitted to EMBL under accession number HG005136.

## RESULTS

### Taxonomic summary

**Phylum:** Microspora (Sprague 1977)
**Class:** Microsporea (Levin & Corliss, 1963)
**Order:** Microsporidia (Balbiani, 1882)
**Suborder** Apansporoblastina (Tuzet, Maurand, Fize, Michel & Fenwick, 1971)
**Family:** Enterocytozoonidae (Cali and Owen, 1990)
**Genus:** *Obruspora* gen. nov.
**Diagnosis:** Monotypic genus with characters of the family Enterocytozoonidae Cali and Owen, 1990. Sporonts and spores develop in direct contact with the host cell cytoplasm, transforming the infected cell into a large xenoma; nuclei are unpaired throughout known developmental stages; meronts not observed; sporogonial plasmodia multinuclear, contain ellipsoidal electron-dense disc-shaped bodies with lucent core that coalesce precociously in the sporont to form turns of polar tube; spores minute, monomorphic, subspherical with a single nucleus; endospore electron lucent, exospore electron dense; polar filament isofilar, coils arranged in several rows; anchoring disc opposite the posterior vacuole at the anterior end of the spore; large, prominent polaroplast; parasites of fish.
**Etymology:** Obruo (Latin) = to overwhelm, obscure. Refers to the nature of the type species, which produces large xenomas that progressively overwhelm the host ovary.

### *Obruspora papernae* sp. nov.

**Diagnosis:** With the characteristics of the genus. Merogonic and sporogonic stages with unpaired nuclei developing in direct contact with host cell cytoplasm. Spores arranged in orderly, paracrystalline arrays; spore mean measurement 1.52 × 1.22 µm (N = 10, with TEM). Polar filament isofilar with 15 to 16 turns, coiled in 3 tiers.
**Type host:** *Callionymus filamentosus* Valenciennes (Teleostei: Callionymidae).
**Additional host:** *Lernanthropus callionymicola* (based on presence of detectable 16S rDNA only).
**Type locality:** Ashdod, Israel, 20 m depth (31° 49′ N, 34° 37′ E to 31° 45′ N, 34° 35′ E).
**Type material deposition: Holotype:** Heavily infected ovary preserved in formalin, deposited at the Steinhardt National Natural History Museum and Research Center, Invertebrate Collection, Catalogue Number TAU-MP-3.
**Paratypes:** Paraffin-embedded histological sections

stained with hematoxylin and eosin; Catalogue Number TAU-MP-4.

**Site of infection:** Ovary. Exact cell type infected uncertain, most likely follicular epithelium.

**Etymology:** Named in honor of the late Prof. Ilan Paperna, eminent Israeli parasitologist, in tribute to his significant contributions to the fields of fish parasitology and fish diseases.

**Gene sequences:** Partial sequence of the 16S rDNA gene is deposited under EMBL accession number HG005137

**Remarks:** Although it is a marine species, *Obruspora papernae* was determined within the Terresporidia or clade IV sensu Vossbrinck & Debrunner-Vossbrinck (2005) containing mostly parasites of terrestrial origin. In view of the details of sporogonial development of the parasite in the host cell cytoplasm, the formation of multiple xenomas, the target organ (ovaries), and the sequence divergence from other available enterocytozoonid sequences, this microsporidium is assigned a novel genus within the family Enterocytozoonidae.

### Host–parasite relationship

*Callionymus filamentosus* was the most abundant fish species in our 2008 to 2012 night trawl landings at a depth of 20 m, comprising nearly 25% of all collected individuals (Table 2). Prevalence and intensity of *Obruspora papernae* were high throughout most months (Table 3). A total of 1491 (38.9%) of the collected *C. filamentosus* individuals were females, of which 1234 (82.7%) had visible microsporidian xenomas. An uninfected female with a normal ovary is shown in Fig. 1a. Both ovarian lobes were typically involved, and the number of xenomas per ovary varied (Fig. 1b–f). In gross examination, heavily affected ovaries were completely overtaken by xenomas (Fig. 1d). The abdomen in such females was distended, and whitish xenomas were visible through the skin (Fig. 1e). Xenomas were found only in ovaries; in 1 case they occurred in the caudal muscle posterior to the visceral mass in an apparently abnormal extension of the ovary (Fig. 1f). Freshly dissected xenomas measured from 0.5 to 4 mm, and upon rupture contained a whitish, opaque fluid.

The ovary of *Callionymus filamentosus* displayed asynchronous development. Developing oogonia and primary oocytes were contained within the paired ovary in 2 layers of follicular cells, enclosed in internal epithelial lamellar folds. In the pre-vitellogenetic phase, the flattened follicular envelope was poorly developed. As they matured, the follicular cells hypertrophied and developed basophilic inclusions. With approaching maturation, vitellogenetic primary oocytes formed cytoplasmic yolk globules and remained closely associated with follicle cells during development, separated by a thin vitelline membrane. A bi-layered envelope covered the ova, an inner zona pellucida made of cube-shaped granulosa cells and outer zona granulosa of elongated thecal cells associated with blood capillaries. In Fig. 2, a growing xenoma is surrounded by oogonia and primary and secondary oocytes.

Table 2. Species forming >1% of individuals caught with trawl nets hauled at a depth of 20 m (night samples; 2008 to 2012) on the Israeli Mediterranean coast

| Species | Relative abundance (%) | Origin |
|---|---|---|
| *Callionymus filamentosus* | 24.61 | Red Sea |
| *Pagellus erythrinus* | 6.21 | Mediterranean |
| *Plotosus lineatus* | 5.24 | Red Sea |
| *Equulites klunzingeri* | 4.68 | Red Sea |
| *Decapterus russelli* | 3.62 | Red Sea |
| *Nemipterus randalli* | 3.25 | Red Sea |
| *Bothus podas* | 2.48 | Mediterranean |
| *Lagocephalus suezensis* | 1.85 | Red Sea |
| *Lithognathus mormyrus* | 1.66 | Mediterranean |
| *Trachurus mediterraneus* | 1.41 | Mediterranean |
| *Engraulis encrasicholus* | 1.06 | Mediterranean |

Most observed lesions were large, well developed xenomas, with a heterogeneous cytoplasm containing randomly dispersed light and dark foci. The microsporidium spores were arranged in arrays (Fig. 3a–c) that stained light blue with H&E. Under TEM, the xenoma spore masses appeared closely packed together in stacked paracrystalline arrays. The microsporidium spores were sub-spherical and measured 1.5 to 2 µm in diameter (Fig. 3d). They were observed in xenomas as well as in cells directly adjoining oocytes, but never inside the germinal cells. The precise cell type targeted by the parasites was not determined, but the location of infected cells suggested they were a component of the follicular epithelium.

A host immune response was evident in some xenomas. Lymphocytes infiltrated the xenoma, and fibroblasts enveloped it peripherally (Fig. 4), with granular areas of immune cell infiltration and early proliferation of fibroblasts. An example of 3 concurrent phases of xenoma development in *Callionymus filamentosus* ovary is shown in Fig. 5a: an active xenoma (X); an advanced stage of degeneration (XG); and a degenerate xenoma transformed into

Table 3. *Callionymus filamentosus.* Blotchfin dragonet data showing prevalence (Prev.) and intensity (Int.; 0 = uninfected, 1 = few small xenomas, 2 = large xenoma patches, 3 = ovary still visible, 4 = ovary tissue obscured) of *Obruspora papernae* n. sp. in the trawl samples collected throughout the study

| Month | All fish (n) | Size group (mm) | Females | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Total (n) | Infected (n) | Prev. (%) | Intensity (n) | | |
| | | | | | | Mean | Uninf. | Int. = 1 |
| **2010** | | | | | | | | |
| Jul | 472 | 54–84 | 4 | 3 | 75 | | 1 | 0 |
| | | 85–114 | 196 | 193 | 98 | | 3 | 19 |
| | | 115–144 | 37 | 37 | 100 | | 0 | 4 |
| | | 145–174 | 6 | 5 | 83 | | 1 | 0 |
| | | *All* | *243* | *238* | *97.94* | *2.3 (135)* | *5* | *23* |
| Nov | 639 | 54–84 | 218 | 194 | 89 | | 22 | 37 |
| | | 85–114 | 16 | 14 | 88 | | 2 | 2 |
| | | *All* | *234* | *208* | *88.89* | *2.6 (100)* | *27* | *39* |
| **2011** | | | | | | | | |
| Jan | 431 | 54–84 | 42 | 39 | 93 | | 3 | 7 |
| | | 85–114 | 102 | 95 | 93 | | 7 | 27 |
| | | 115–144 | 2 | 2 | 100 | | 0 | 1 |
| | | *All* | *146* | *136* | *93.15* | *2.4 (84)* | *10* | *35* |
| Mar | 1184 | 54–84 | 109 | 70 | 64 | | 39 | 28 |
| | | 85–114 | 330 | 275 | 83 | | 55 | 53 |
| | | 115–144 | 10 | 10 | 100 | | 0 | 4 |
| | | *All* | *449* | *355* | *79.06* | *2.3 (335)* | *94* | *85* |
| May | 477 | 54–84 | 19 | 6 | 32 | | 13 | 4 |
| | | 85–114 | 225 | 129 | 57 | | 96 | 48 |
| | | *All* | *244* | *135* | *55.33* | *2 (134)* | *109* | *52* |
| Oct | 294 | 85–114 | 35 | 34 | 97 | | 1 | 14 |
| | | 115–144 | 60 | 57 | 95 | | 3 | 6 |
| | | 145–174 | 5 | 4 | 80 | | 1 | 1 |
| | | *All* | *100* | *95* | *95.00* | *2.3 (95)* | *5* | *21* |
| **2012** | | | | | | | | |
| Jan | 335 | 54–84 | 61 | 55 | 90 | | 8 | 10 |
| | | 85–114 | 14 | 12 | 86 | | 2 | 0 |
| | | *All* | *75* | *67* | *89.33* | *2.9 (67)* | *10* | *10* |
| **Total** | 3832 | 54–84 | 453 | 367 | 81 | | 84 | 86 |
| | | 85–114 | 918 | 752 | 82 | | 166 | 176 |
| | | 115–144 | 109 | 106 | 97 | | 3 | 15 |
| | | 145–174 | 11 | 9 | 82 | | 2 | 1 |
| | | *All* | *1491* | *1234* | *82.76* | | *255* | *278* |

granuloma (G). While females displayed ovaries packed with the parasites and only remnants of ovarian cells remaining wedged in between the xenomas (Fig. 5b), all male gonads examined with histology (N = 10) displayed the typical testicular structure with crypts of normally developing sperm (Fig. 5c).

### Transmission electron microscopy (TEM)

The xenoma nucleus was hypertrophic and multilobed, and sections of it could be seen in various areas of the structure. All developmental stages of the microsporidium were in direct contact with the host cell cytoplasm.

The earliest observed developmental stages of *Obruspora papernae* were divisional sporogonial plasmodia (Fig. 6a–c). These contained round sporonts surrounded by numerous ellipsoid, disc-like, membrane-bound vesicles that measured approximately 80 × 250 nm and appeared to divide by plasmotomy (arrows; Fig. 6d). The disc-like vesicles appeared to coalesce, forming the polar tube of the primordial extrusion apparatus. Additional stages of formation and organization of the polar tube were observed in more advanced developmental stages of the sporogonial plasmodium (Fig. 6e–h). In some

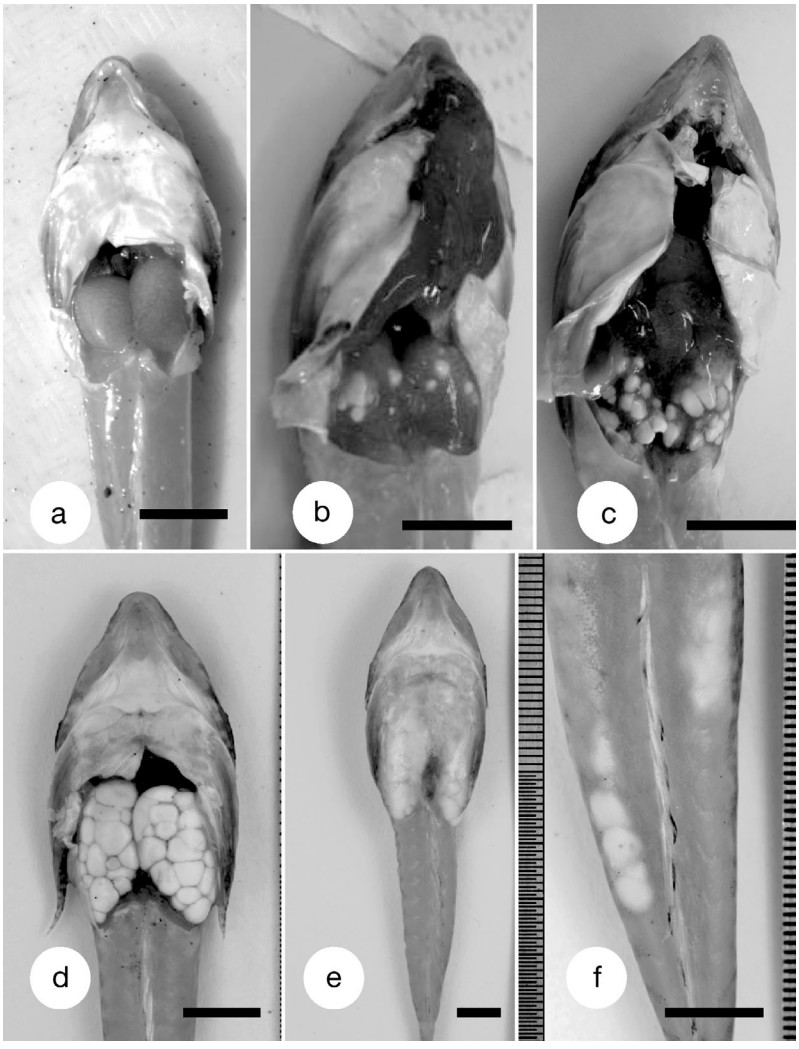


Fig. 1. *Callionymus filamentosus* infected by *Obruspora papernae.* Clinical signs of xenomas in female blotchfin dragonet. (a) Normal female with uninfected ovary. Xenomas in (b) light and (c) moderate infections. (d) Exposed viscera in a heavily infected female, showing virtual takeover of the ovary by the parasite. (e) A heavily infected female, with xenomas visible through abdominal skin. (f) Anomalous extension of infected ovary into the caudal muscle, with xenomas visible through skin. Scale bars = 10 mm

cases, clusters of maturing sporoblasts included forms that displayed aberrant development (Fig. 6i). Individual spores were in direct contact with the host cell cytoplasm (Fig. 6j).

Developing *Obruspora papernae* sporoblasts and spores occurred both in xenomas and cells recognized as follicular epithelium (Fig. 7a). The cytoplasm of the latter contained unidentified elongated, electron lucent areas subdivided by thin membranes with dark nucleus-like inclusions (Fig. 7b). In xenomas, spherical bodies of an unknown nature measuring 1 to 1.5 µm were observed (Fig. 7c). Sporoblasts at various stages of maturation could be observed side by side (Fig. 7d). Young spores of *O. papernae* contained a single nucleus, anchoring disc, polaroplast, posterior vacuole, and a clear demarcation of an electron dense exospore surrounded by an electron lucent endospore (Fig. 7e). At high power magnification, the 15 to 16 isofilar polar tube coils arranged in 3 tiers could be discerned (Fig. 7f).

### Histological and molecular analyses of *Lernanthropus callionymicola*

Specimens of *Lernanthropus callionymicola* were removed from the gills of *Callionymus filamentosus*. Mean (±SD) body length (excluding fourth legs, N = 415 females, N = 62 males) was 1.1 ± 0.12 mm (range 1.3–2.8 mm) for males and 2.0 ± 0.26 mm (0.8–1.44 mm) for females. Copepod females often had 2 egg sacs. Gross examination of copepods revealed no discoloration, inclusions, or any other noticeable abnormalities, nor did histological sections reveal any pathological features. However, PCR products from the 5′ region of the *Obruspora papernae* rDNA sequence were successfully amplified from total DNA extract of *L. callionymicola* copepods which originated from 2 different localities in Ashdod (Israel) and Iskenderun (Turkey) (Fig. 8) and from both infected and non-infected hosts.

### Molecular phylogeny

The ML phylogenetic tree is presented in Fig. 9. The ML and Bayesian topologies were largely similar and only differed in the positioning of weakly supported nodes. The phylogenetic analysis agrees with the results of Freeman & Sommerville (2009) and Nylund et al. (2010). *Obruspora papernae* clusters inside the family Enterocytozoonidae with high support (BP = 97; PP = 1). Within the Enterocytozoonidae, it is placed within a well supported clade (BP = 93; PP = 0.99) which contains 3 de-

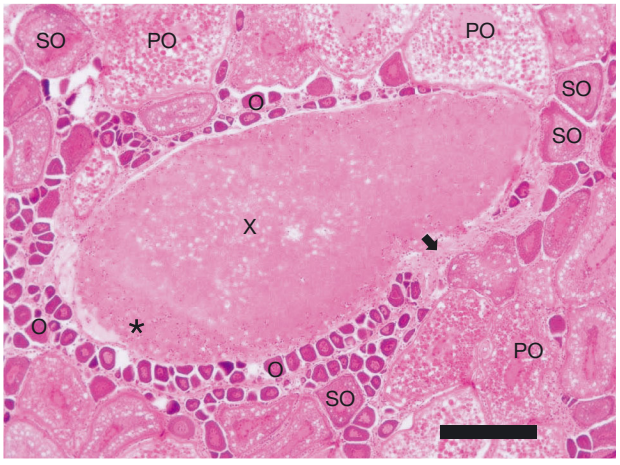


Fig. 2. *Obruspora papernae.* An early xenoma (X) surrounded by oogonia (O), secondary (SO) and primary (PO) oocytes. Note distinct granular area (*) with infiltration of host immune cells. Early proliferation of fibroblasts is seen at the xenoma periphery (arrow). Scale bar = 200 µm

scribed species (*Nucleospora salmonis*, *N. cyclopteri*, and *Desmozoon lepeophtherii*) as well as 3 sequences from 2 undescribed microsporidian species: 1 from the eel *Anguilla rostrata* (JN938583) and 1 from the English sole *Parophrys vetulus* (AF186007 and AF201911; Gresoviac et al. 2000, Khattra et al. 2000) (BP = 93; PP = 0.99). Relationships within this clade are not supported and vary depending on the phylogenetic analysis except for the monophyly of *N. salmonis* + *N. cyclopteri* (BP = 99; PP = 0.99), and a close relationship of the English sole parasite with *D. lepeophtherii* (BP = 100; PP = 1).

Sequence divergence between the *Obruspora papernae* sequence and other sequences in the Enterocytozoonidae clade ranges between 8 and 18%. These values exceed within-genera divergence observed in microsporidia and are in the range of divergence observed between closely

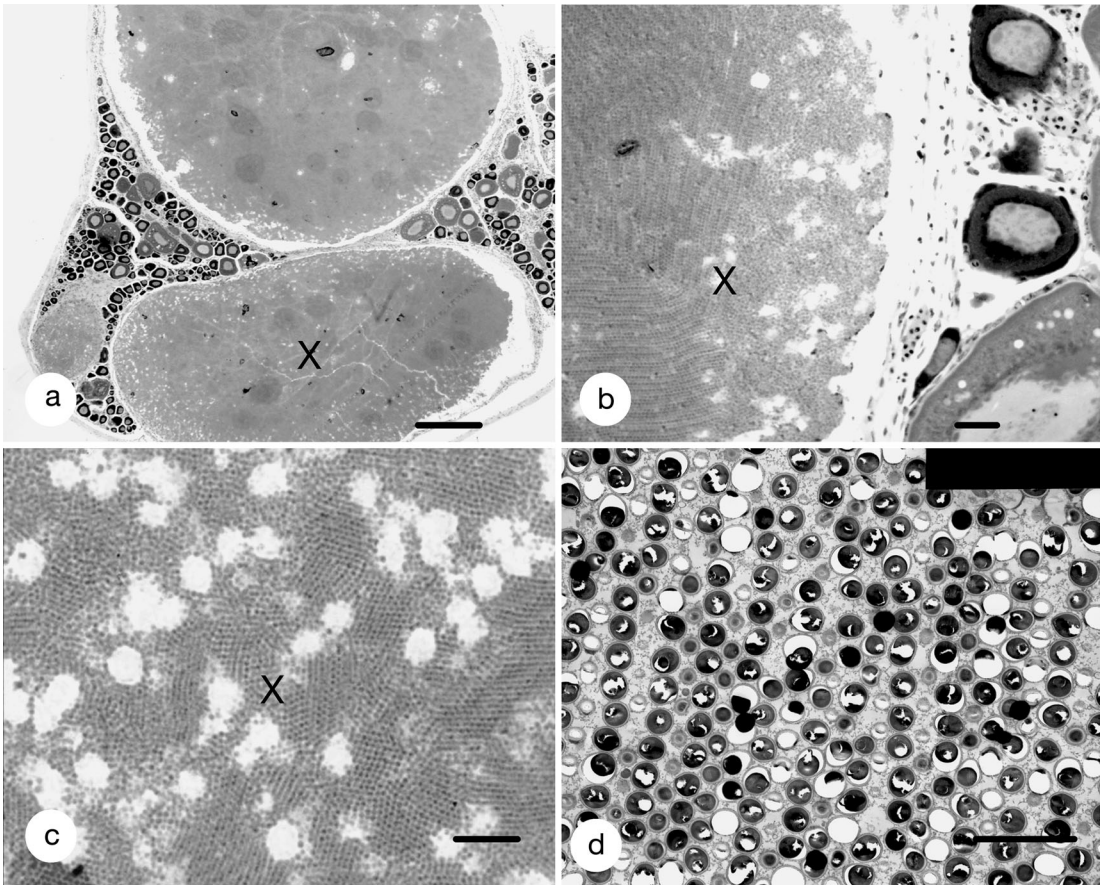


Fig. 3. *Callionymus filamentosus* infected by *Obruspora papernae.* Xenomas (X) in blotchfin dragonet ovary. (a) Low-power magnification showing heterogeneous internal contents with foci of dispersed dark-staining cytoplasm. (b) Periphery of xenoma showing cytoplasm rich with spores. (c) Spores arranged in paracrystalline arrays within the xenoma. (d) TEM micrograph showing paracrystalline array of spores. Scale bars = (a) 250 µm, (b) 40 µm, (c) 25 µm, (d) 5 µm

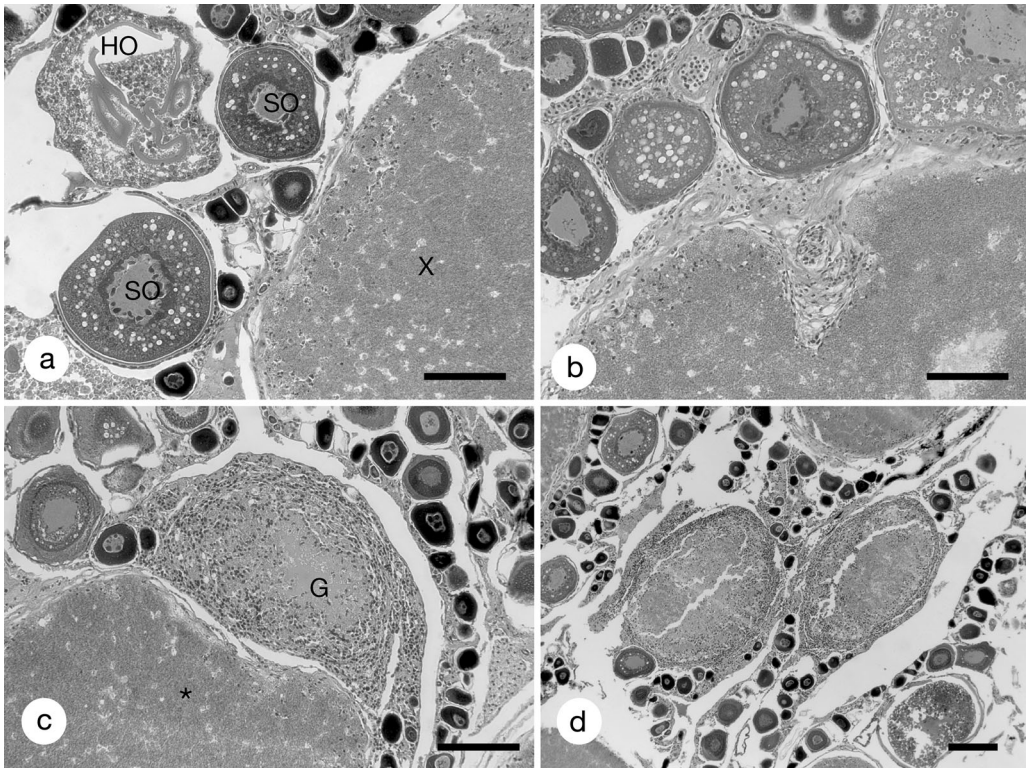


Fig. 4. *Obruspora papernae.* (a) Xenoma (X) periphery showing mature, hydrated (HO) as well as secondary oocytes (SO) and oogonia. (b) Xenoma with invagination of fibroblasts. (c) Granulomatous xenoma (G) adjacent to active xenoma (*). (d) Granulomatous remnants of 2 degenerative xenomas (XG). All scale bars = 100 µm

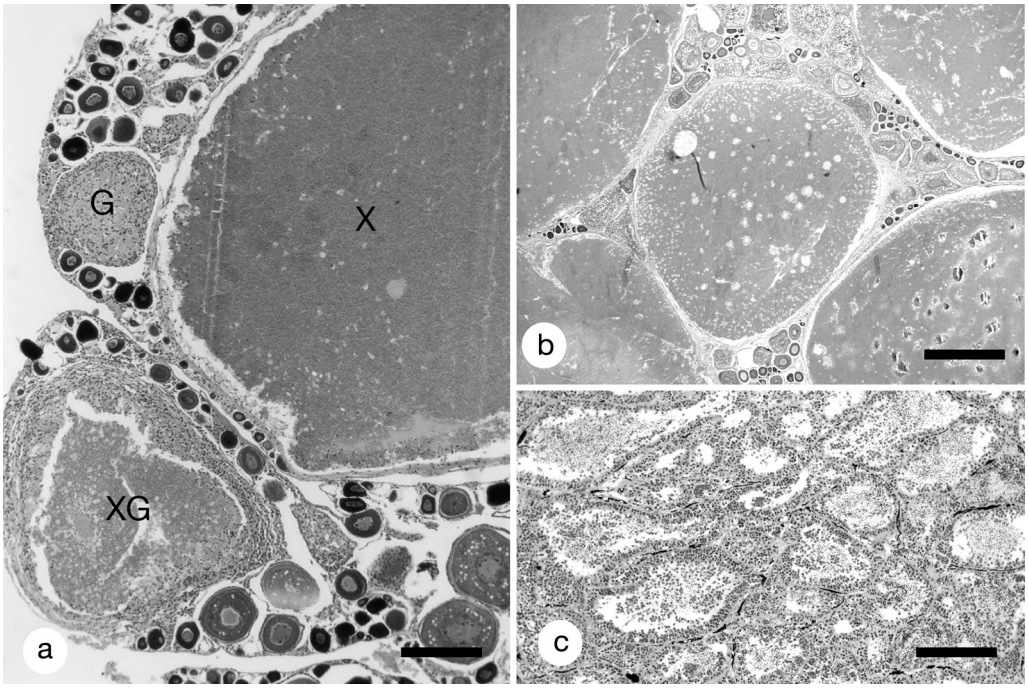


Fig. 5. *Callionymus filamentosus* infected by *Obruspora papernae.* (a) Three phases of xenoma development in blotchfin dragonet ovary. An active xenoma (X), a xenoma in the process of degeneration (XG), and a granuloma (G). (b) Severely infected ovary overwhelmed with xenomas; some vestigial remnants of germ tissue are seen wedged in between the xenomas. (c) Uninfected gonad of male dragonet showing normal development of sperm. Scale bars = (a,c) 200 µm, (b) 1 mm

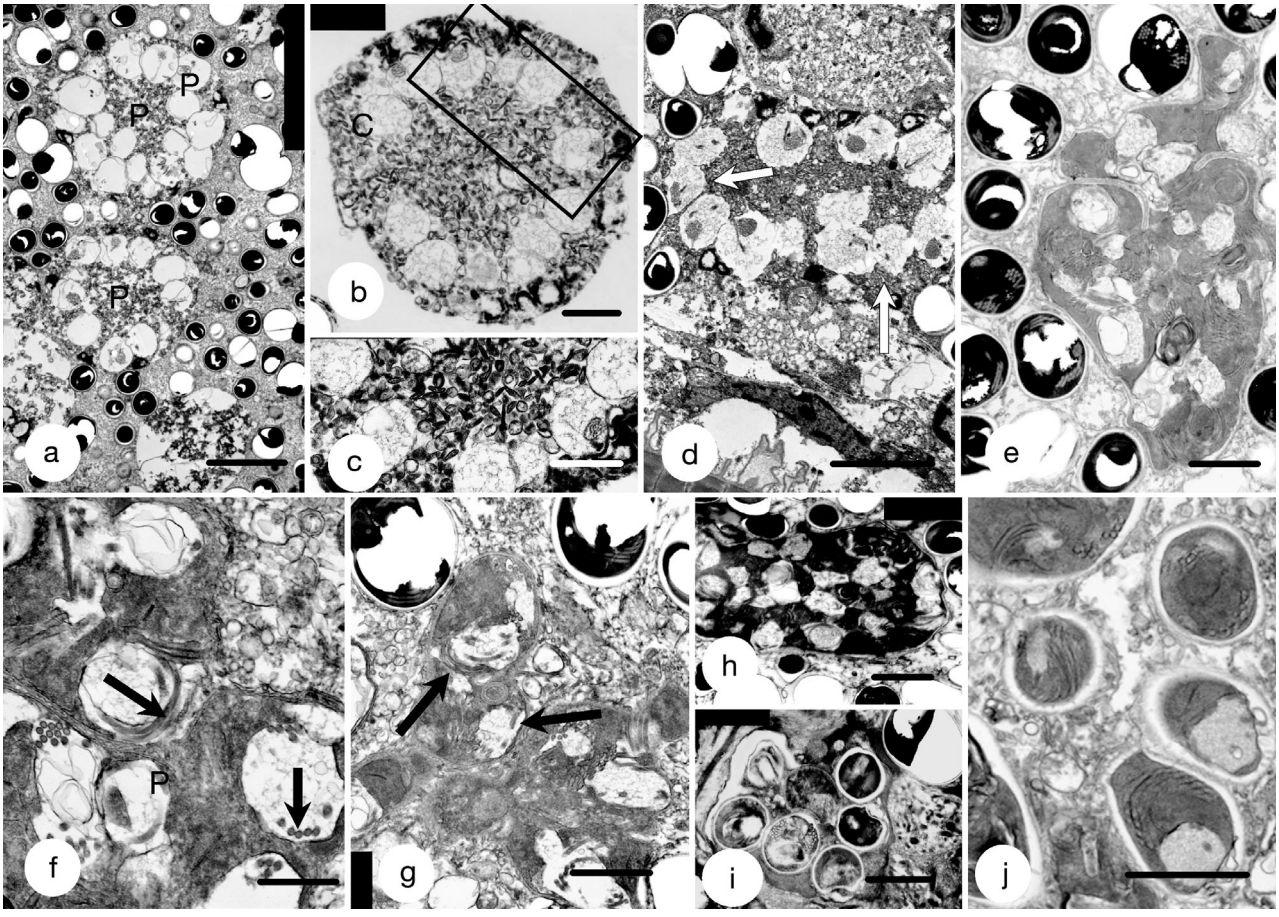


Fig. 6. *Obruspora papernae.* Development. (a) Early sporogonial plasmodia (P) surrounded by mature spores; ellipsoid, disc-like vesicles can be seen inside the plasmodium. (b) Sporogonial plasmodium. (c) Higher magnification of box in (b), showing the disc-like vesicles. (d) Sporogonial plasmodium (P) with developing sporonts showing peripheral nuclei. Some of the sporonts appear to be undergoing plasmotomy (arrows), including disc-like vesicles at an early stage of polar tube organization. (e) Sporogonial plasmodium with sporonts at an advanced stage of developing polar tube. (f,g) Maturing sporoblasts, showing primordial polar tube coils (arrows). (h) Advanced sporogonic plasmodium. (i) Aberrantly developing sporoblasts. (j) Maturing spores showing polaroplast, anchoring disc, posterio vacuole, exospores, and endospore. Scale bars = (a,d) 5 µm, (b,e,f,g,j) 1 µm, (c) 1.5 µm, (h,i) 2 µm

related genera (e.g. Diamant et al. 2010). As a case in point, sequence divergence between *Desmozoon* and *Nucleospora* ranges between 10 and 21%, while within *Nucleospora* it ranges from 1 to 15%.

### Historical data

The data obtained from museum collection specimens of *Callionymus filamentosus* dating back to 1974 are presented in Table 4. A proposed timeline of the invasion of *C. filamentosus* and its 2 associated parasites into the eastern Mediterranean is presented in Fig. 10.

## DISCUSSION

Microsporidian xenomas are thought to develop in fish from immune system phagocytes (Lom & Dyková 2005). Although lymphocytes and macrophages were among the interstitial cell types observed in the ovary, the cell type targeted by the microsporidium appeared to be a component of the follicular epithelium (FE) of *Callionymus filamentosus*. FE cells function in the transport of nutrients, yolk proteins, and metabolites between the oocytes and the bloodstream, and are known to possess phagocytic capacities (Hunter & Macewicz 1985). Following the observations of Phelps & Goodwin (2007) on *Ovipleistophora*

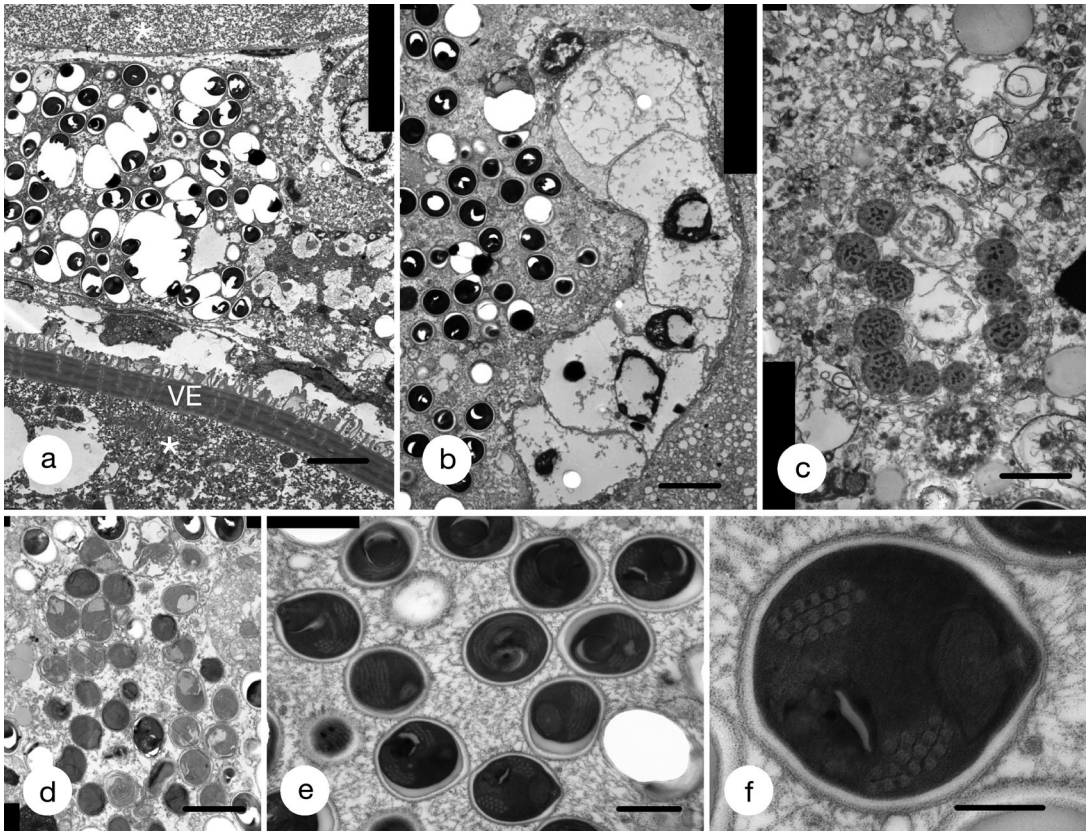


Fig. 7. *Obruspora papernae.* (a) Mature spores and sporoblasts developing in an ovarian epithelial cell, positioned in between 2 oocytes (asterisks); VE: vitelline envelope of oocyte. (b) Mature spores in the cytoplasm next to an unidentified electron lucent body containing dark nucelus-like inclusion. (c) Unidentified spherical bodies within the xenoma. (d) Cytoplasmic region within xenoma showing active sporgony. (e) Maturing spores. (f) A single spore. Scale bars = (a,b,d) 5 µm, (c) 2 µm, (e) 1 µm, (f) 0.5 µm

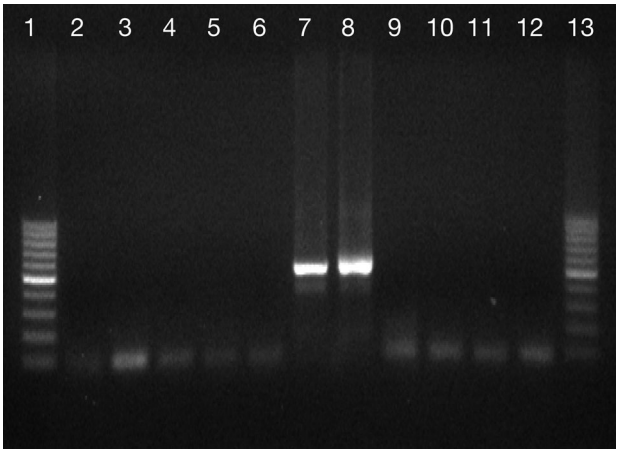


Fig. 8. PCR gel showing occurrence of the parasite *Callionymus* in *Lernanthropus callionymicola* copepods. The 5' region of the parasite rDNA sequence was amplified from total DNA extract of *Lernanthropus* copepods collected from the gills of *Callionymus filamentosus*. Lanes 1 & 13: 100 bp size-ladder. Lanes 2–11: Copepods from Ashdod (Israel) or Iskenderun (Turkey). Lane 12: Negative control. PCR results were reproducible

*ovariae*, we suggest that such phagocytic FE cells were the most likely targets of *Obruspora papernae*, since infections did not develop in the male gonads (which lack follicles).

*Obruspora papernae* gen. et sp. nov. xenomas impact the host ovary by reducing the volume available for functional germinal tissue as well as by exerting physical pressure on the surrounding parenchyma. In addition, deleterious metabolites that originate in the xenoma are probably released into the surrounding tissue, inducing cell membrane damage (Lom & Dyková 2005). Judging from the gross pathology and histology features, there is a progressive, long-term detrimental effect on the ovary. However, the question regarding what effect the xenomas have on egg production is intriguing. There are various existing reports on microsporidium infections in gonads of both genders (e.g. Sanders et al. 2012). In *Oncorhynchus tshawytscha* (Walbaum, 1792) spores of *Loma salmonae* (Putz, Hoffman and Dunbar, 1965) may be found in ova as well

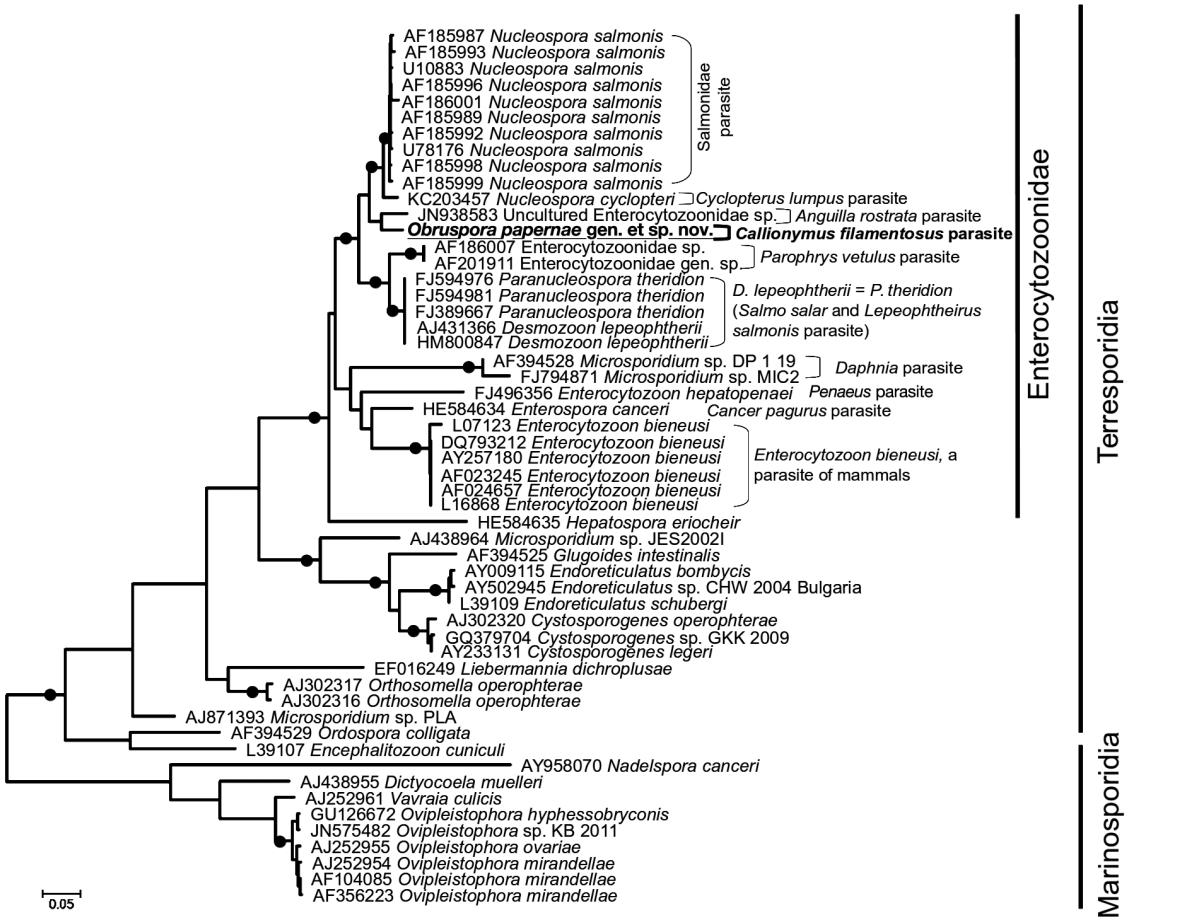


Fig. 9. Bayesian phylogenetic tree reconstructed from microsporidian rRNA sequences. Host taxa are indicated next to each clade. Branch support at the nodes is maximum likelihood bootstrap percentage (BP)/ Bayesian posterior probability (PP). Only branches with support higher than 50/0.5 are presented

as in xenomas developing on ovarian blood vessels (Docker et al. 1997). *O. papernae* induces xenomas only in female *Callionymus filamentosus,* but the parasite does not invade the ova itself, unlike other gonadotropic microsporidia. For example, *O. ovariae* develops inside ova, which presumably facilitates vertical transmission. In some host species, spores are released into the ovary during spawning activity (e.g. Summerfelt & Warner 1970, Docker et al. 1997).

The high prevalence of *Obruspora papernae* xenomas throughout much of the year suggests that at least part of the parasite burden is retained between spawning seasons. *Callionymus filamentosus* females seem to retain a degree of fecundity throughout infection, since oocytes and oogonia were observed even in very heavily infected ovaries. The dimensions of *C. filamentosus* ripe ova were 0.3 to 0.4 mm, significantly smaller than the xenomas (0.5 to 4.0 mm). The capacity of the infected gonad to produce ova, in the end, would be inversely correlated with the ovarian volume occupied by the xenomas. Liberation of xenomas during spawning would probably alleviate some of the internal visceral pressure in swollen abdomens (Casal & Azevedo 1995), but could also lead to exposure of progeny to parasite spores through contaminated ovarian fluid (Sanders et al. 2012).

Dragonets are gonochoristic batch spawners (Breder & Rosen 1966), and fertilization is external in 3 studied species, *Callionymus maculatus* Rafinesque, 1810, *C. ornatipinnis* Regan, 1905, and *Bathycallionymus kaianus* (Günther, 1880) (Johnson 1973, Ikejima & Shimizu 1999, Awata et al. 2010). It is most likely similar in *C. filamentosus*. Spore dispersal is probably more effective in batch spawners, since external shedding of mature spores and xenomas (intact or ruptured) will occur over an interval rather than as a one-time event, as in the 'total spawner' strategy. Creation of fish aggregations would facilitate exposure of naive individuals to released spores and promote new infections during the spawning season.

Table 4. Occurrence of parasite infections in museum specimens of dragonets (*Callionymus filamentosus*, *Diplogrammus randalli*) collected between 1974 and 2005 (prev.: prevalence; int.: intensity). Copepods *Lernanthropus callionymicola* were first found on the gills of Mediterranean dragonets collected in 1997 while microsporidian lesions in Mediterranean dragonet ovaries due to *Obruspora papernae* were first noted in samples collected in 2004. Neither of the parasites was found in Red Sea museum specimens. ND: (sex) not determined; TL: total length; TAU: Steinhardt National Natural History Museum and Research Center, Tel Aviv University; HUJ: Hebrew University Zoological Museum

| Species | TL range (mm) | Collection date (d.mo.yr) | Locality | n | Sex M | Sex F | *L. callionymicola* Prev. (%) | *L. callionymicola* Max. int. | *O. papernae* Prev. (%) | *O. papernae* Max. int. | Museum cat. no. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *D. randalli* | 40–83 | 27.09.1974 | Ras Garra, Gulf of Suez | 30 | ND | | 0 | 0 | 0 | 0 | TAU P.6258 |
| *C. filamentosus* | 122–146 | 28.08.1977 | Off Bardawil | 6 | 6 | 0 | 0 | 0 | 0 | 0 | TAU P.9830 |
| *C. filamentosus* | 113–125 | 28.09.1977 | Off Bardawil | 2 | 0 | 2 | 0 | 0 | 0 | 0 | TAU P.9830 |
| *D. randalli* | 89 | 22.11.1977 | Ras Garra, Gulf of Suez | 1 | 1 | 0 | 0 | 0 | 0 | 0 | TAU P.6848 |
| *C. filamentosus* | 125–144 | 21.08.1978 | Off Bardawil | 4 | 1 | 3 | 0 | 0 | 0 | 0 | TAU P.6745 |
| *C. filamentosus* | 86–112 | Summer 1978 | Off Bardawil | 3 | 0 | 3 | 0 | 0 | 0 | 0 | TAU P.9251 |
| *D. randalli* | 44–78 | 24.09.1981 | Foul Bay (south), Tiran Island | 9 | ND | | 0 | 0 | 0 | 0 | TAU P.9031 |
| *C. filamentosus* | 82–150 | 30.03.1983 | Jaffa | 26 | 4 | 22 | 0 | 0 | 0 | 0 | HUJ.13997 |
| *C. filamentosus* | 103–121 | 02.01.1987 | Haifa | 10 | 9 | 1 | 0 | 0 | 0 | 0 | HUJ.12096 |
| *C. filamentosus* | 66–89 | 27.02.1987 | Palmachim | 6 | ND | | 0 | 0 | 0 | 0 | TAU P.9601 |
| *C. filamentosus* | 71–75 | 28.02.1987 | Nitzanim | 3 | 0 | 3 | 0 | 0 | 0 | 0 | TAU P.9603 |
| *C. filamentosus* | 63–78 | 15.04.1987 | Haifa | 5 | 0 | 5 | 0 | 0 | 0 | 0 | HUJ.12245 |
| *C. filamentosus* | 43–87 | 05.11.1987 | Palmachim | 3 | ND | | 0 | 0 | 0 | 0 | TAU P.9787 |
| *C. filamentosus* | 100 | 20.10.1988 | Palmachim | 1 | ND | | 0 | 0 | 0 | 0 | TAU P.10113 |
| *C. filamentosus* | 79–127 | 19.09.1997 | Palmachim | 16 | 1 | 15 | 87.5 | 8 | 0.0 | 0 | TAU P.11233 |
| *C. filamentosus* | 75–130 | 20.09.2004 | Palmachim | 16 | 2 | 4 | 68.8 | 11 | 68.8 | 4 | TAU P.13090 |
| *C. filamentosus* | 65–125 | 23.09.2005 | Palmachim | 10 | 0 | 10 | 80.0 | 7 | 90.0 | 4 | TAU P.13219 |

Although teleost tissue reactions to microsporidia have been extensively studied (Lom & Dyková 2005, Kent et al. in press), the interaction of the xenoma with the surrounding host tissue is still poorly understood; in particular, the precise signal that triggers and mobilizes leukocytes, attracting them to the xenoma is unknown. Existing evidence points at the innate immune system, particularly inflammatory components such as macrophages, lymphocytes, neutrophils, and eosinophilic granular cells, as well as fibroblasts that participate in xenoma obliteration and eventual spore elimination. However, initiation of the process is apparently dependent on xenoma size, once maximum development has been reached (Rodriguez-Tovar et al. 2011).

The xenomas in *Callionymus filamentosus* show similarities to previously described infections in fish ovary, such as those associated with *Ichthyosporidium* spp. in *Clevlandia ios* (Jordan & Gilbert, 1882) (Sanders et al. 2012). Each young individual xenoma is surrounded by a thin envelope, presumably originating from a single hypertrophic cell. It has a spherical shape and in it, the parasite develops to produce mature spores. This corresponds with the '*Glugea*-xenoma' type described by Dyková & Lom (1980), characterized by true xenoma formation and eventual xenoma demise in a granulomatous reaction. Three stages based on host tissue reactions were characterized by Dyková & Lom (1980): weak, productive, and xenoma involution; all xenomas are eventually obliterated by the host immune reaction. The phagocytic immune cells putatively gain access into the xenoma through gaps that form in the outer membrane and proceed to destroy the spores (Rodriguez-Tovar et al. 2011). In *C. filamentosus*, we observed all 3 stages of xenoma containment, infiltration of phagocytic leukocytes, and overlay of fibroblasts and formation of a granulomatous envelope.

## Phylogenetic affinities

*Obruspora papernae* shares attributes with other members of the Enterocytozoonidae; the developmental sequence has similarities with species of *Nucleospora* as well as *Paranucleospora*/*Desmozoon* and *Enterocytozoon* spp., in particular the vesicular polar tube precursors (also termed 'electron-dense disc-like structures' or EDDs; see Desportes-Livage et al. 1996) that coalesce to form the extrusion apparatus primordium at the early stage of sporont development. *O. papernae* has minute, nearly spherical

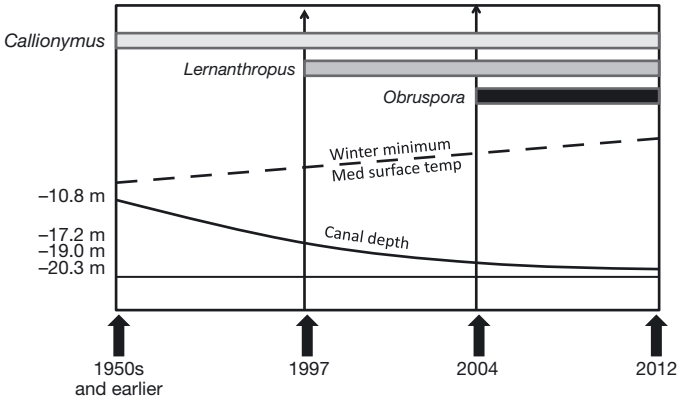


Fig. 10. Timeline of appearance of *Callionymus filamentosus* and its 2 parasites in the eastern Mediterranean. The emergence of the microsporidium *Obruspora papernae* occurred 7 yr after the first record of the copepod ectoparasite *Lernanthropus callionymicola* and over 5 decades after its fish host was initially documented. During that period, the Suez Canal was deepened and widened, and a minimum winter surface temperature rise took place in the eastern Mediterranean. Time axis not to scale

spores (Fig. 7). Fish microsporidium spores are typically oval or ellipsoid and generally form disorganized spore aggregations (e.g. Canning & Curry 2005, Diamant et al. 2010). *O. papernae* forms vast, unusual paracrystalline, 3-dimensional spore arrays with a lattice-like appearance. To the best of our knowledge, this is a unique feature that has not been previously reported in the Microsporidia.

*Paranucleospora/Desmozoon* has 3 developmental cycles, 1 in the copepod host *Lepeophtheirus salmonis* (Krøyer, 1837) and 2 in Atlantic salmon *Salmo salar* Linnaeus, 1758 (Freeman & Sommerville 2009, Nylund et al. 2010). So far, only 1 developmental cycle has been found in the *Callionymus filamentosus* microsporidium. It specifically targets the host's ovary where it induces large xenomas, while *D. lepeophtherii* in salmon induces no xenomas and targets gills and internal organs, but not gonads. However, *Desmozoon* does induce subcuticular, epidermal xenomas in its copepod host (Freeman & Sommerville 2009). Two of the life cycles of *D. lepeophtherii* take place in the host cell cytoplasm, while 1 is intra-nuclear. The spore polar tube of *D. lepeophtherii* is short and anisofilar, while in the *C. filamentosus* microsporidium, it is long and isofilar. These distinctions, together with the differences in morphology, ultrastructure, the results of the phylogenetic analysis, and the different biogeographical regions lead us to conclude that it is a distinct taxonomic entity that warrants the erection of a new genus for which we propose the name *Obruspora*.

## Biogeographical and ecological attributes

To date, 700 alien metazoan species — macrophytes, invertebrates, and fish — have been recorded in the Mediterranean. In terms of magnitude, frequency, and duration of propagule transfer, the Suez Canal has by far superseded any other pathway for a large number of successfully established alien populations in the Mediterranean Sea, with over 80% of the current alien species documented from the Israeli coast having gained access through this route (Galil 2012). In the present study, we examined this unique phenomenon from the perspective of a long established invasive fish population and its newly emerged castrating microsporidian parasite.

Invasive species are confronted with numerous challenges in their new environment and many fail to establish (Hatcher & Dunn 2011). Hypotheses have been offered as to why some succeed while others do not; recently, Gaither et al. (2013) attempted to predict the spread of introduced marine species by the nature of population structure in their native range. The question why some parasites are successfully introduced with their host while others are not is complex due to factors including host specificity, host immunity, and complexity of life cycles, rendering the result of host and parasite species mixing unpredictable. The emergence of new parasites and diseases has received increased attention in recent years; however, many aspects of host–parasite system function under newly encountered environmental conditions are still relatively poorly understood (Benmayor et al. 2009, Hatcher & Dunn 2011). The enemy release hypothesis argues that host establishment is facilitated by release from natural enemies and parasites that have been left behind (Torchin et al. 2003), but when tested in experimental field studies, results have been equivocal. It was found that the parasite load was not simply reduced per se, and that in some cases, abundance of successfully invading parasites actually increased their intensity of infection to levels that were significantly higher than those found in their native range (Hines et al. 1997, Innocenti & Galil 2007, 2011, Pasternak et al. 2007).

*Callionymus filamentosus* has been living in the Mediterranean for at least 60 yr (Ben Tuvia 1953). Only recently have individuals been found to harbor the microsporidium *Obruspora papernae,* long after the invading host populations had become established, increased in numbers, and dispersed along the Levant sub-littoral (Golani & Bernardi 2012). Despite the high-level infections by the castrating parasite, the host has remained abundant and its

populations are enormous; it is the most abundant fish species represented in shallow trawl fishery landings on the Israeli Mediterranean coast (Stern 2010; see Fig. 1).

Parasitic castration is distinguished from fecundity reduction, which is a host strategy in which energy is temporarily diverted from reproduction to be invested in a more urgent task, such as immunity (Hurd 2001, Lafferty & Kuris 2009). Parasitic castrators are important regulators of host population density since they inflict 'reproductive death' on their hosts (Baudoin 1975, Lafferty & Kuris 2009) which drives a reduction in overall host density (Lafferty & Kuris 1996). *Callionymus filamentosus* and its parasite *Obruspora papernae* do not seem to follow this tenet, since the continued abundance of the host suggests that despite compromised female fecundity the species is overcoming parasite impact. *O. papernae*-infected hosts undergo gradual reduction of fecundity as xenoma volume increases, until most or all of the ovarian tissue is replaced. Presumably the parasite would benefit from maintaining a degree of host fertility since continued spawning and egg discharge that most likely also promotes shedding of parasite spores would ensure host–parasite system continuity. *C. filamentosus* and its castrating parasite have clearly reached a steady state of large host population size coupled with consistently high parasite prevalence. Counter-strategies that compensate for population loss have been known from host–parasite systems challenged by castrating parasites. These include elevated host fecundity, early host sexual maturation, temporary increase of reproductive effort, or induced elongation of the spawning season (see Forbes 1993, Lafferty & Kuris 2009). Another invasive species of Red Sea origin that has invaded the Mediterranean is the swimming crab *Charybdis longicollis* Leene, 1938, accompanied by its sacculinid parasite *Heterosaccus dollfusi* Boschma, 1960. Here too, despite castrating its host, *H. dollfusi* has exhibited 20 successive years of high prevalence in the Mediterranean (Innocenti & Galil 2011). It was suggested that the highly fecund crustacean host with 'open' recruitment dynamics, and its rhizocephalan parasite with 'closed' recruitment dynamics, were sufficient to maintain an adequately high population density for upholding high infection levels (Innocenti & Galil 2007).

Hosts may alter their foraging patterns in attempt to circumvent the effects of a castrating parasite. When infected by the tapeworm *Hymenolepis diminuta* (Rudolphi, 1819) the beetle *Tenebrio molitor* (Arai, 1980) will increase its total food intake, particularly carbohydrates. This dietary modification has been experimentally shown to counteract reduction in fecundity: the augmented nutrition is commensurate with the increased demands of the developing parasite (Ponton et al. 2011). A different strategy would be to shift to earlier host reproduction mode, to preempt the parasite's virulence later in life, with expected curtailment of the host's future reproductive output. This strategy has been observed in trematode-infected snails, where compensation for anticipated loss in reproductive success is countered by increased egg deposition shortly after exposure to the parasite (Minchella & Loverde 1981), and also in hosts infected by microsporidia. *Glugoides intestinalis* (Chatton, 1907) castrates the freshwater cladoceran *Daphnia pulex* Leydig, 1860; upon infection, the host shifts towards increased early production of offspring, generating in the first clutch nearly 40% more offspring than uninfected hosts (Chadwick & Little 2005). Similarly, the invasive freshwater gammarid *Dikerogammarus villosus* (Sowinsky, 1894) increases production of eggs following exposure to the castrating microsporidian parasite *Cucumispora dikerogammari* Ovcharenko and Kurandina, 1987 (Bacela-Spychalska et al. 2012). In fish, the intraovarian microsporidia *Ovipleistophora ovariae* (Summerfelt, 1964) and *O. mirandellae* (Vaney & Conte, 1901) (Summerfelt & Warner 1970, Canning & Lom 1986) may reduce host fecundity by up to 40%. In fact, *O. ovariae* infections in golden shiner *Notemigonus crysoleucas* (Mitchill, 1814) may result in larger spawners (as compared to uninfected spawning individuals), suggesting that microsporidian infections may redirect resources towards body growth (Summerfelt & Warner 1970). How *Callionymus filamentosus* counteracts parasite-mediated fecundity loss is yet to be determined.

Freshwater microsporidia have been known to accompany their invasive hosts to new regions (Slothouber Galbreath et al. 2004, Ovcharenko et al. 2010), but we are unaware of any parallel case in marine hosts. A suspected invasive alien microsporidium that induces severe infections in a Levantine population of the common stingray *Dasyatis pastinaca* (Linnaeus, 1758) was investigated by Diamant et al. (2010); however, the origin of that parasite is unknown. Invasive ectoparasitic copepods originating in the Red Sea have switched hosts ('spilled over') to native Mediterranean host fish (El Rashidy & Boxshall 2010). In the present study, we were unable to unequivocally determine whether *Obruspora papernae* gen. et sp. nov. is a natural parasite of *Callionymus filamentosus* in its native Red Sea region, or

a locally acquired Mediterranean species picked up through spillback (sensu Kelly et al. 2009). Nevertheless, circumstantial evidence suggests that *O. papernae* is also an invasive species: (1) no ovarian xenomas similar to those it induces have been documented in any other fish in the Mediterranean Sea; (2) the high prevalence and intensity of infection, coupled with low pathogenicity and weak host immune response, would indicate a co-evolutionary history; (3) finally, the observed decline in *O. papernae* prevalence and intensity in colder months in the Mediterranean suggests it is a thermophilic species, as is characteristic of invasive species introduced through the Suez Canal (Ben Tuvia 1966, Galil 2012).

Vertically transmitted parasites are typically avirulent and their transmission is independent of host density (Dunn & Smith 2001). Thus, vertically transmitted alien parasites are naturally pre-adapted to evade selective pressures leading to enemy release (Slothouber-Galbreath et al. 2010). Since microsporidia can transmit between hosts in both horizontal and vertical (transovarian) routes (Dunn & Smith 2001), it follows that they have an increased likelihood of being present during invasion of their natural host. Indeed, they tend to be retained during invasion of their hosts: native and alien host populations of the freshwater gammarid amphipod *Cragonyx pseudogracilis* Bousfield, 1958 were found to harbor identical microsporidium species in both native and naturalized habitats (Slothouber-Galbreath et al. 2010). Nevertheless, since successful establishment is dependent on a variety of factors, 'non-permissive' conditions to a given parasite may persist for an extended period while its host establishes a 'parasite-free' population. We suggest that the successful establishment of *Obruspora papernae* in the Mediterranean Sea took place only recently, years after its host had established a population, possibly as a result of 2 major changes, viz. a rise in local sea surface temperatures and expansion of the Suez Canal.

The Mediterranean mean annual sea surface temperatures have increased by up to 1.5°C in the last 2 decades (Raitsos et al. 2010), and it is not unusual for small changes in temperature to significantly alter parasite survival (Poulin 2006). Fish microsporidia are known to be temperature sensitive (Antonio & Hedrick 1995, Sveen et al. 2012), and low temperatures retard and even arrest development in some species (Olson 1981, Zenke et al. 2005). In the microsporidium *Loma salmonae,* temperatures below 9°C interrupt the sporogonial development and production of xenomas (Beaman et al. 1999). In *Cucumispora dikerogammari,* an increase in prevalence during summer in its host populations (*Dikerogammarus villosus*) in the Baltic Sea has been linked with increased host foraging rates, enhanced probability of encountering infected prey, and boosted parasite development due to elevated temperature (Bacela-Spychalska et al. 2012). Increase of annual mean sea surface temperatures in the Levant basin may have had a critical effect on the emergence, development, and transmission of fish parasites along the Israeli coast. In the last 20 yr, 2 important low-temperature sensitive seawater parasites have emerged: the ectoparasitic ciliate *Cryptocaryon irritans* and the myxosporean *Enteromyxum leei* (Diamant et al. 1991, Diamant 1992). Both are thermophilic species that display suppression of growth during low winter temperatures, while summer temperatures bring about high prevalences and proliferation in Mediterranean marine aquaculture facilities (Diamant et al. 1991, Diggles & Lester 1996, Estensoro et al. 2010). Our field data on *Obruspora papernae* xenomas indicates high-intensity infections in *Callionymus filamentosus* during much of the year, with a temporary drop during the colder months (January to May).

The rise in Mediterranean sea surface temperatures co-occurs with an enlargement of the Suez Canal. The canal has undergone several expansions over the past 2 decades to sustain the increasing number and dimensions of vessels. The typical cross-sectional area of the canal increased from 304 $m^2$ in 1869 to 1800 $m^2$ in the 1970s, to 3600 $m^2$ in 2000, and at present is 5200 $m^2$ (www.suezcanal.gov.eg/sc.aspx?show=12). The prevailing current in the canal is directed northwards, and increased water volumes moving through the canal have obvious implications on propagule transport, as the recent surge of Red Sea species invasion into the Mediterranean reveals (Galil 2012). We suggest that the massive influx of Erythraean biota probably included *Callionymus filamentosus* propagules which were infected with *Obruspora papernae*. White & Perkins (2012) proposed a model that predicts asymmetries arising between native and alien populations of invasive host species. In the new range, due to parasite release (or to loss of specific alleles at immune loci, due to the bottleneck effect), an invading population may experience reduced immunogenic diversity leading to reduced immunity to the missing parasites. This model offers a scenario that corresponds with *C. filamentosus* invasion in that early invaders of the Mediterranean, which were missing *O. papernae* for decades, gradually lost resistance since natural selection would function

to re-allocate resources away from costly (and now unnecessary) immune defenses, in favor of increased growth and reproduction. Regardless, selective forces working on the host immune system against a castrating parasite would presumably be aimed at prevention of infection rather than maintenance and coping with it (Lafferty & Kuris 2009). Thus, as long as the parasite was absent, a trade-off could be expected. However, when a subsequent set of invasive host propagules brought in the missing parasite — according to the model of White & Perkins (2012) — it would enable spillover to the now 'receptive' established host population, producing high-prevalence infections. The model also suggests that the parasite, at the early invasive stage, would be expected to be under r-selection pressure for increased transmission. This model therefore provides an explanation as to the rapid spreading of *O. papernae* throughout a receptive Mediterranean *C. filamentosus* host population, as was observed in our study.

A final point that warrants elucidation is the role, if any, of the ectoparasitic copepod *Lernanthropus callionymicola* in the development and spread of the microsporidian parasite. Assuming that *L. callionymicola* was introduced with its host into the Mediterranean (El Rashidy & Boxshall 2012), its earliest Mediterranean record is a specimen of *Callionymus filamentosus* collected in 1997, 7 yr before the documentation of the first infection of *Obruspora papernae* (Table 4). The timing of appearance of the 2 *C. filamentosus* parasites may not be coincidental since the fish host had established its populations over half a century ago, and yet neither parasite was reported for many years thereafter. We were unable to detect any evidence of *O. papernae* developmental stages in *L. callionymicola*. However, the repeated finding of microsporidian rDNA in copepod individuals removed from the host gills is significant. While it could simply suggest ingestion of infected fish tissue by the ectoparasite, it may indicate an as yet undetermined role of the copepod in the life cycle of the microsporidium, as observed in ectoparasitic copepods of salmonids (Freeman & Sommerville 2009, Nylund et al. 2010, Jones et al. 2012).

Elucidating the mechanisms that control alien parasite–host systems in their newly invaded areas is gaining increasing interest as part of the current attempt to better understand the effects and impacts of anthropogenic activities on bioinvasions and corresponding links with emerging diseases (Hatcher et al. 2012). Since parasites may have a significant impact on the development and survival of the host, some play a crucial role in the fate of their invasive host (Prenter et al. 2004), and our study clearly illustrates the unpredictable outcome of bioinvasions.

The apparent discrepancy between the castrating parasite's impact at the individual host level and its apparent absence of expression at the population level adds to the questions raised by an earlier study of the *Charybdis–Heterosaccus* host–parasite system (Innocenti et al. 2009): both exemplify the poorly understood complexity of host–parasite systems, constantly adapting, co-evolving, and co-responding to an ever-changing environment (the Red Queen Hypothesis; e.g. Roth et al. 2012).

*Obruspora papernae* was therefore probably introduced through the Suez Canal like its host *Callionymus filamentosus*. The course of its emergence in the Levant is somewhat similar to that previously observed in the population of the invasive rhizocephalan sacculinid *Heterosaccus dollfusi* (Innocenti & Galil 2007). Both parasites appeared decades after their respective hosts established large populations in the Mediterranean and both have a castrating effect on their hosts. However, not only are the parasites and hosts from different phyla, whereas in *C. filamentosus* only females mass produce the parasite, in *Charybdis longicollis* both sexes are castrated and propagate the parasite. Furthermore, the sacculinid induces significant morphological and behavioral changes in its host (Galil & Lützen 1995, Innocenti et al. 1998, 2003). No similar phenomena were observed in *C. filamentosus.*

The effect of parasitic castrators on their host population is considered to be so strong that their use as biological pest control agents has been proposed (Lafferty & Kuris 2009). According to theoretical models incorporating the negative association between host density and prevalence of parasitic castrators, it would be expected that the rapid spread of *Obruspora papernae* in the established Mediterranean populations of *Callionymus filamentosus* would lead to a decline in host population density. However, the field data from the present study of an alien microsporidium/fish parasite–host pair differs from theory-based patterns (e.g. Antonovics 2009). This corresponds with the results reported for the 20 yr accumulation of data from the studies on the aforementioned alien sacculinid/decapod crustacean pair (Innocenti & Galil 2011). Future empirical studies will hopefully elucidate the inherent plasticity of host–parasite systems and how this resilience is expressed by host species facing fresh challenges with old parasites in new environments.

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