MOLECULAR ECOLOGY

Molecular Ecology (2013) **22**, 314–326 doi: 10.1111/mec.12126

Single-nucleotide polymorphisms of two closely related microsporidian parasites suggest a clonal population expansion after the last glaciation

KAREN L. HAAG,* EMMANUEL TRAUNECKER† and DIETER EBERT*

Zoological Institute, University of Basel, Vesalgasse 1, CH-4051 Basel, Switzerland, †Department of Biomedicine, University of Basel, Hebelstrasse 20, CH-4031 Basel, Switzerland

Abstract

The mode of reproduction of microsporidian parasites has remained puzzling since many decades. It is generally accepted that microsporidia are capable of sexual reproduction, and that some species have switched to obligate asexuality, but such process had never been supported with population genetic evidence. We examine the mode of reproduction of *Hamiltosporidium tvaerminnensis* and *Hamiltosporidium magnivora*, two closely related microsporidian parasites of the widespread freshwater crustacean *Daphnia magna*, based on a set of 129 single-nucleotide polymorphisms distributed across 16 genes. We analyse 20 *H. tvaerminnensis* isolates from localities representative of the entire species' geographic distribution along the Skerry Island belt of the Baltic Sea. Five isolates of the sister species *H. magnivora* were used for comparison. We estimate the recombination rates in *H. tvaerminnensis* to be at least eight orders of magnitude lower than in *H. magnivora* and not significantly different from zero. This is corroborated by the higher divergence between *H. tvaerminnensis* alleles (including fixed heterozygosity), as compared to *H. magnivora*. Our study confirms that sexual recombination is present in microsporidia, that it can be lost, and that asexuals may become epidemic.

Keywords: asexuality, clonality, microsporidia, mode of reproduction, parasite, recombination, single-nucleotide polymorphism

Received 18 April 2012; revision received 3 October 2012; accepted 7 October 2012

Introduction

The occurrence of sexual reproduction in microsporidia, a diverse group of minute unicellular and intracellular parasites closely related to Fungi (James *et al.* 2006), has been a longstanding, unsolved issue (Hurst *et al.* 1992; Flegel & Pasharawipas 1995; Hurst 2000; Lee *et al.* 2010b). Sexual reproduction in pathogenic organisms is of public health interest, because it facilitates the spread of mutations (e.g. mutations that confer drug resistance) throughout populations, by creating novel genetic combinations on which selection can act (Barton & Charlesworth 1998; Barton 2010). In *Toxoplasma gondii*, for example, epidemic clones seem to originate via asexual amplification of highly virulent genotypes generated by rare events of sexual reproduction (Sibley & Ajioka 2008). There are several examples of unicellular parasites that only rarely engage in sexual reproduction, such as *Trypanosoma brucei* (Koffi *et al.* 2009) and *T. gondii* (Sibley & Ajioka 2008). However, obligately asexual pathogenic species are rare, since sexual reproduction enables responses to environmental challenges in nature, in the host or in response to antimicrobial therapy (Heitman 2006). Some fungal pathogens, such as *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* that were considered asexual, have recently been shown to undergo cryptic sexual or parasexual cycles (Heitman 2010). The degree to which pathogens experience sexual reproduction is often associated with the complexity of their life cycles. In rust fungi, some species alternate between asexual and sexual reproduction

Correspondence: Karen L. Haag, Fax: +41 61 267 03 62;
E-mail: karen.haag@unibas.ch

in different species of host plants, such as the poplar rust (*Melampsora larici-populina*; Barrès *et al.* 2012) that reproduces sexually on larch and multiplies asexually on poplar, and others are obligately asexual, relying on a single-host species, for example, the wheat yellow rust (*Puccinia striiformis*; Hovmøller *et al.* 2002; Enjalbert *et al.* 2005) and the wheat leaf rust (*Puccinia triticina*; Goyeau *et al.* 2007).

Genomic studies coupled with population genetic analyses are an indirect but powerful way of assessing reproductive modes, which are usually difficult to uncover in microorganisms. For example, using fluorescence *in situ* hybridization performed with probes to an episomal plasmid, sex has only recently been documented in *Giardia*, a protozoan parasite of humans (Poxleitner *et al.* 2008). The full description of a sexual process in *Giardia* was preceded by the identification of a complete set of meiotic genes (Ramesh *et al.* 2005), and a population genetic survey showing single-nucleotide polymorphisms (SNPs) shuffled throughout distinct chromosome regions (Cooper *et al.* 2007). However, recombination *per se* does not constitute sufficient evidence for the prevalence of sexuality (Rich *et al.* 1997; Birky 2010). Sexual reproduction might be rare, or virtually absent, and still leave the footprint of recombination in the genome (Balloux *et al.* 2003; de Meeûs & Balloux 2004). In *Giardia*, that undergoes an unusual ploidy cycle, it remains unknown whether recombination results from a meiotic or a parasexual cycle, when it occurs, or how common it is (Birky 2010).

In diploid species, asexuality can be inferred from population genetic data (Tibayrenc *et al.* 1990; Tibayrenc & Ayala 1991; Halkett *et al.* 2005): first, in fully asexual populations heterozygosity is fixed; second, in obligately asexual species, the two alleles harboured by any individual at a locus will diverge from each other due to the lack of recombination, and divergence is proportional to the time since asexuality started. The latter is known as 'allele sequence divergence' (ASD; Birky 1996) or 'Meselson effect' (Judson & Normark 1996). The Meselson effect leads to higher than expected coalescence times of intraindividual alleles and is expected over long timescales in clonal lineages (Ceplitis 2003). Higher than expected amounts of heterozygosity have been used to argue for a predominantly asexual mode of reproduction in *C. albicans* (Névavi *et al.* 2006; Bougnoux *et al.* 2008), *P. triticina* (Goyeau *et al.* 2007) and *T. brucei* (Koffi *et al.* 2009), but so far, there is no evidence for ASD in putatively asexual parasites (Birky 2010). Strong ASD was found in endomycorrhizal fungi, which are thought to have been asexual for approximately 400 Myr (Kuhn *et al.* 2001; Pawlowska & Taylor 2004; Bakker *et al.* 2010).

There are more than 1200 described microsporidian species (Mathis 2000), infecting a wide range of animals, including many of economic importance, such as fish and honey bees. In humans, microsporidia are opportunistic pathogens that emerged as a health problem since the AIDS pandemic and were added to the National Institute of Allergy and Infectious Diseases (NIAID) priority pathogen list. Despite their importance, we know little about their population genetic structure and mode of reproduction. Current descriptions of sexual processes in microsporidia are based only on ultrastructural observation and are restricted to a well-studied group that parasitizes mosquitoes (e.g. *Amblyospora*, *Edhazardia*; Hazard & Brookbank 1984; Sweeney *et al.* 1988, 1990; Chen & Barr 1995), which has received attention for its potential to control mosquito vector populations (Sweeney & Becnel 1991). For most microsporidians, however, the existence of sexual reproduction remains an open question.

It has been suggested that microsporidians possess a system of sex determination similar to fungi (Lee *et al.* 2008). Besides having a conserved synteny of several genes, a MAT-type locus resembling the one described for a taxon of primitive fungi (Zygomycota; Idnurm *et al.* 2008) was found in the genome of three microsporidian species, *Encephalitozoon cuniculi*, *Enterocytozoon bieneusi* and *Antonospora locustae* (Lee *et al.* 2008). As opposed to fungi, however, there is no evidence for idiomorphism (segregation of two highly divergent types of alleles) in the MAT-type loci of any of these microsporidian species (Lee *et al.* 2010a). Clues of sexuality in microsporidians stem from observations about cell structure and development. For example, some species (e.g. *Vairimorpha* spp) show diplokaryotic merogonial stages that are followed by a sporogonial stage, which often leads to eight monokaryotic spores, suggesting that sporogony is preceded by meiosis (Canning *et al.* 1999). Synaptonemal complexes observed during sporogony, followed by plasmogamy in *Amblyospora* spp have been interpreted as evidence for meiosis and sexual reproduction (Hazard & Brookbank 1984; Hazard *et al.* 1985; Sweeney *et al.* 1990; Chen & Barr 1995). Conversely, the lack of concerted evolution of rRNA genes, in combination with the absence of a monokaryotic stage in some microsporidian species (Gatehouse & Malone 1998; Tay *et al.* 2005; O'Mahony *et al.* 2007) has been taken as evidence for asexuality. With indirect evidence based on life cycle observations and the developmental patterns of several microsporidian species, it was suggested that sexual reproduction is ancient and that asexuality may have originated independently several times in microsporidia (Ironside 2007). Similar to fungi, microsporidia that are considered to be obligately asexual complete their life cycle in a single host,

whereas those that show elements of asexual and sexual reproduction in their spore ultrastructure often require two different hosts (Andreadis 2007).

Hamiltosporidium tvaerminnensis is a microsporidian parasite that specifically uses the cladoceran *Daphnia magna* as a host, and it has only been found in the intermittent rock–pools of the Skerry Islands in the Baltic Sea (Haag *et al.* 2011). *H. tvaerminnensis* differs from the sister species, *H. magnivora*, by its mode of transmission, whereas the former is transmitted both horizontally and vertically, the latter has never been shown to transmit horizontally among *Daphnia,* but may have a second, yet unidentified, host (Mangin *et al.* 1995 – in this article *H. tvaerminnensis* was called *Tuzetia* sp). We suspect that the second host is required for completing the sexual cycle. Here, we investigate the mode of reproduction in these two sister species of microsporidia based on SNPs. We take advantage of the recently published genome draft sequence of *H. tvaerminnensis* (previously named *Octosporea bayeri*; Corradi *et al.* 2009), to look for population genetic evidence of recombination and sexual reproduction. We sequenced 16 loci in the genome of 20 isolates sampled from locations across the entire known geographic distribution of this species. We compare our findings with data from a Belgian population of *H. magnivora*, and conclude that, differently from its sister species, *H. tvaerminnensis* is asexual, a condition that might have been key for its success in colonizing Fennoscandia after the last Ice Age.

Materials and methods

On the Skerry Islands of the Baltic Sea, approximately 50 per cent of the *D. magna* populations are infected by *H. tvaerminnensis* (Ebert *et al.* 2001), and parasite prevalence within infected populations can reach 100 per cent during summer (Lass & Ebert 2006). Its sister species *H. magnivora* had previously been included in the genus *Flabelliforma*, but differs considerably from the *Flabelliforma*-type species, *F. montana* (Haag *et al.* 2011). *H. magnivora* seems to be less specialized regarding its hosts than *H. tvaerminnensis* (being also found in *Daphnia pulex* and *Daphnia longispina*); it also has a broader geographical distribution, having been found in Central and Eastern Europe and the Middle East. In spite of that maximal prevalence of *H. magnivora* in *D. magna* populations seems to be rather low (around 6% in two populations in Belgium; Decaestecker *et al.* 2005; E. Decaestecker, unpublished).

Sampling, PCR and sequencing

The *H. tvaerminnensis* genome draft, deposited at NCBI as a whole-genome shotgun project with accession no. ACSZ00000000, was searched (BLASTN and TBLASTX) for sequences that could be used as population genetic markers. Sixteen genomic regions covering 10 721 bp were used to screen for SNPs. Genomic regions included nine housekeeping genes (alpha-tubulin, beta-tubulin, hsp70, acetyl-transferase, kinesin, chaperonin, karyopherin, pyruvate-dehydrogenase and phosphoglucose-isomerase) and seven sequences encoding a GTP-binding protein, a ribosomal protein, a hypothetical protein, different regions of a SKT5 protein, a high-mobility group protein and a spore wall protein (see Table S1 for details).

Our sampling strategy of *H. tvaerminnensis* was designed to cover its entire known geographic range (Table 1). We analysed 20 isolates of *H. tvaerminnensis*, and five *H. magnivora* isolates from Belgium, obtained between August of 2006 and August of 2010. To our knowledge, the geographic ranges of the two *Hamiltosporidium* species do not overlap. Each isolate consisted of total DNA purified from a single infected *D. magna* using the DNeasy extraction kit (QIAGEN). Infections were verified by phase contrast microscopy at 400× magnification.

All PCRs were performed in a volume of 50 μL containing 1.25 U of *Taq* Polymerase (Sigma), 1X reaction buffer, 1.5 mM $MgCl_2$, 20 pmol of each primer (Microsynth), 200 μM dNTP and 100–500 ng of template DNA. The PCR profile consisted of an initial denaturation step at 95 °C for 5 min, over 35 amplification cycles (annealing at 50 °C for 30 s, extension at 72 °C for 60 s, denaturation at 95 °C for 30 s) and a final extension at 72 °C for 5 min. Amplicons were column purified (Sigma) and sequenced directly (Macrogen, South Korea and Microsynth, Switzerland).

Sequence analyses, polymorphism estimates

Nucleotide sequences were assessed for quality and assembled with CodonCode Aligner version 3. Some sequences showed double peaks in regions of high-quality reads, which we interpreted as heterozygous polymorphisms (SNPs). A few chromatograms are presented in Fig. S1, illustrating how sequencing double peaks have been scored as heterozygotes. We generated multiple alignments where sites showing double peaks were represented by IUPAC ambiguity codes. To estimate species-specific parameters of polymorphism, an alignment containing all SNPs (Table S2) was phased using the unphase tool implemented in DnaSP version 5 (Librado & Rozas 2009). We used this data set to calculate the number of segregating sites (s), the average number of pairwise nucleotide differences (k), nucleotide diversity (π), Tajima's D and the minimum number of recombination events (Rm). Another data set was

Table 1 Geographic coordinates of the *Hamiltosporidium* sampling locations, from West to East

Species	Isolate	Location	Coordinates
Hamiltosporidium Tvaerminnensis	SE-G1-1	Island nr.1 near Gräsö Island, Sweden	60°25′ N, 18°30′ E
	SE-G2-3	Island nr.2 near Gräsö Island, Sweden	60°25′ N, 18°31′ E
	SE-G4-20	Island nr.4 near Gräsö Island, Sweden	60°24′ N, 18°34′ E
	FAT-1-1	Aland Island, Finland	60°01′ N, 19°54′ E
	FSE-2-45	Martinkari, near Selii, Turku region	60°15′ N, 22°01′ E
	SML-57	Smultrongrundet, Hanko, Finland,	59°49′ N, 22°58′ E
	SML-28	Smultrongrundet, Hanko, Finland	59°49′ N, 22°58′ E
	OER-3-3	Ören, Tvärminne, Finland	59°47′ N, 23°10′ E
	IS1-6	Isskär, near Tvärminne, Finland	59°49′ N, 23°11′ E
	Ob13	Fyrgrundet, near Tvärminne, Finland	59°49′ N, 23°14′ E
	Ob16	Fyrgrundet, near Tvärminne, Finland	59°49′ N, 23°14′ E
	G-42	Granbusken, near Tvärminne, Finland	59°48′ N, 23°14′ E
	G-43	Granbusken, near Tvärminne, Finland	59°48′ N, 23°14′ E
	N-18-1	Storgrundet, near Tvärminne, Finland	59°49′ N, 23°15′ E
	BR1-39	Branskär, near Tvärminne, Finland	59°50′ N, 23°16′ E
	SEG-9	Segelskär, near Tvärmine, Finland	59°45′ N, 23°22′ E
	LAG-5	Långgrundet, near Tvärminne, Finland	59°84′ N, 23°23′ E
	LAG-9	Långgrundet, near Tvärminne, Finland	59°84′ N, 23°23′ E
	HEL1-3-4	Island near Helsinki	60°08′ N, 24°59′ E
	FHS 1-8-1	Island near Russian border	60°16′ N, 27°12′ E
Hamiltosporidium magnivora	Fm K1-1	Knokke, Belgium	51°20′ N, 3°20′ E
	Fm T1-2	Leuven, Belgium	50°49′ N, 4°35′ E
	Fm T1-3	Leuven, Belgium	50°49′ N, 4°35′ E
	Fm4	Heverlee, Belgium	50°52′ N, 4°41′ E
	Fm7	Heverlee, Belgium	50°52′ N, 4°41′ E

generated with genotypic data of unknown phases to calculate the mean observed and expected heterozygosities (H_O and H_E) over all SNPs within each species with the Arlequin software version 3 (Excoffier *et al.* 2007). Observed and expected heterozygosities were then compared with the fixation index (F) for each population, which was tested for significance using a X^2 distribution (see Hedrick 2011, p. 96).

Recombination estimate

To assess recombination rates, haplotypes of the three most variable markers (alpha-tubulin, beta-tubulin and hsp70) were inferred using Phase version 2 (Stephens *et al.* 2001). The method is based on a Markov chain Monte Carlo scheme that samples from the probability distribution of haplotypes (H) and recombination parameters (R) for a given genotype (G; Pr H,R$^{|}$G). To increase confidence of haplotype estimation, 2–3 isolates from each species had their haplotypes determined experimentally. PCR amplification of each marker was performed using *Pfu* DNA polymerase (Promega), amplicons were cloned using the TOPO cloning kit (Invitrogen), and sequenced with universal primers M13 Forward and M13 Reverse. Haplotypes determined experimentally were included as known data in the Bayesian analysis, which ran over 10 000 iterations using the default recombination model. This allowed us to obtain reliable estimates of the recombination parameter ρ (= $4N_ec$, where N_e is the effective population size, and c is the recombination rate) for each of the three markers in each species (Li & Stephens 2003; Crawford *et al.* 2004).

Allele sequence divergence

Two alleles in a diploid asexual lineage should diverge from each other at a faster rate than in a sexual species, due to the lack of recombination (Birky 1996; Judson & Normark 1996). To evaluate ASD, genetic distances between alleles from each locus (alpha-tubulin, beta-tubulin and hsp70) as well as the concatenated sequences from all three loci were calculated for each *Hamiltosporidium* isolate with Mega 5 (Tamura *et al.* 2011). Genetic distances obtained for *H. tvaerminnensis* and *H. magnivora* were compared by the Wilcoxon rank-sum test using JMP 9. Maximum-likelihood allele phylogenies were inferred for each locus separately using the Kimura 2-parameter model, and considering a gamma distribution of substitution rates among sites (α = 1) and a class of invariable sites. Tree topologies were tested using bootstraps of 1000 replicates.

Flow cytometry

As the identification of haploid spores from meiosis would provide further evidence of sexual reproduction in *Hamiltosporidium*, we investigated the occurrence of meiosis in 40-day-old and six 10-day-old *Hamiltosporidium*-infected *D. magna* from laboratory cultures. Hosts (3–6 individuals) were washed in nuclease-free water to remove the medium and homogenized in 100 μL of phosphate-buffered saline. The homogenate was added on top of 1 mL of a 50% Ludox solution and centrifuged for 15 min at 16 000 ***g***. The spore pellet was washed with phosphate-buffered saline, centrifuged again for 2 min and resuspended in 200 μL methanol for permeabilization. After a short (2 min) incubation, spores were centrifuged for 2 min. The pellet was resuspended in 10 μL of 0.05 mg/mL DAPI (4′,6-diamidino-2-phenylindole) and incubated in the dark for 10 min. The excess DAPI was removed with another phosphate-buffered saline washing.

Spores were analysed on a CyAn cytometer (Beckman Coulter) with three lasers and nine colours. DAPI-stained DNA was excited by the Violet 405 nanometer laser, and the emitted light was captured on FL6 channel (450/50 Filter). Digitalized information was analysed using the Summit 4.3 software (Beckman Coulter). Cell cycle is represented on a histogram showing DAPI Area parameter gated on forward and side scatter population, where debris are separated from spore populations.

Results

DNA polymorphism

A total of 10 721 bp of 20 *H. tvaerminnensis* and five *H. magnivora* isolates were screened for polymorphisms (Table 2), among which 129 SNPs and five indels were found. As shown on the bottom of Table 2, only six SNPs were variable among the 20 *H. tvaerminnensis* isolates (*Pol*), whereas 90 SNPs and five indels were polymorphic within the *H. magnivora* sample. Twelve other SNPs appeared in fixed heterozygosity, but only in *H. tvaerminnensis* (*FHet*). From the total of 129 SNPs, 116 were homozygous (*Hom*) within *H. tvaerminnensis*, whereas only 39 showed fixed homozygosis within the *H. magnivora* sample. Figure 1 shows the character states of a subset of 34 alphatub, betatub and hsp70 SNPs (shaded in Table 2) in isolates from each species; these markers were chosen for recombination analyses (see below) because they showed the largest amount of nucleotide variation, with polymorphism in both species and fixed heterozygosity in *H. tvaerminnensis.* All six *H. tvaerminnensis* variable SNPs were silent mutations. Four of these six mutations are transitions, from which three are singletons, found in isolates FHS 1-8-1 and SEG-9. The other three *H. tvaerminnensis* polymorphic sites were due to the loss of heterozygosity; one in FHS 1-8-1 within hsp70 and two in five isolates within the spore wall protein-encoding gene (SWP25) (for

Table 2 Number of single-nucleotide polymorphisms within 16 *Hamiltosporidium* genes

			Hamiltosporidium tvaerminnensis			*Hamiltosporidium magnivora*		
	Bp	SNPs	*Pol*	*FHet*	*Hom*	*Pol*	*FHet*	*Hom*
Alphatub	783	8	0	3	5	5	0	3
Betatub	701	8	1	2	5	6	0	2
Hsp70	868	18	1	7	10	14	0	4
GBP	507	38*	0	0	38	19*	0	19
ACT	975	9	0	0	9	8	0	1
KIN	643	3	0	0	3	2	0	1
CHAP	569	6	1	0	5	5	0	1
RPL24E	514	2	1	0	1	1	0	1
KAR	508	6	0	0	6	5	0	1
PYDH	743	4	0	0	4	4	0	0
PGI	490	1	0	0	1	1	0	0
EHyPr	508	4	0	0	4	4	0	0
Con18	659	0	0	0	0	0	0	0
Con297	769	4	0	0	4	4	0	0
HMG	773	16	0	0	16	11	0	5
SWP25	711	7	2	0	5	6	0	1
Total	10 721	134*	6	12	116	95*	0	39

Pol, polymorphic within species; *FHet*, heterozygous within species, with fixed heterozygosity; *Hom*, homozygous within species, but divergent between species.
*Includes five indels.

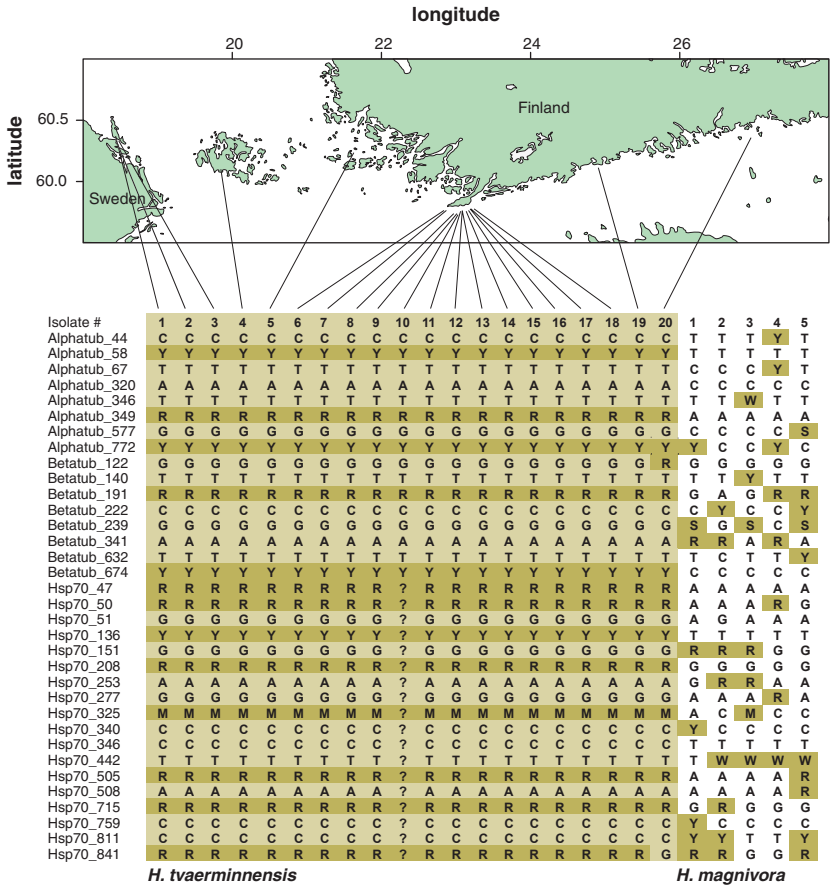

Fig. 1 Character states in a subset of 34 alpha-tubulin, beta-tubulin and hsp70 SNPs. The ambiguous character states (heterozygous) are represented by IUPAC ambiguity codes and are shown on a dark yellow background (M = A/C; R = A/G; W = A/T; S = C/G; Y = C/T; K = G/T). On light yellow background, are the *Hamiltosporidium tvaerminnensis* homozygous character states, and on white background, those of *Hamiltosporidium magnivora*. The map on top shows the geographic distribution of our 20 *H. tvaerminnensis* isolates, which covers the entire species range.

details, see Table S2). SWP25 showed the largest amount of variation within *H. tvaerminnensis* (two SNPs in 711 bp, see Table 2).

Despite the fact that the *H. tvaerminnensis* sample was much larger and came from a broader geographic area, all estimates of DNA polymorphism in *H. tvaerminnensis* were about one order of magnitude smaller than those of *H. magnivora* (Table 3). Furthermore, we observed a highly significant excess of heterozygotes (H_O = 0.80; F = -0.83, Table 3) and a positive and significant Tajima's D (2.81; $P < 0.01$; Table 3) in *H. tvaerminnensis*. Because asexual reproduction is known to increase the coalescence times of alleles within populations (Ceplitis 2003), these data suggest that *H. tvaerminnensis* is largely clonal. Moreover, the minimum number of recombination events estimated from this data set was 2 for *H. tvaerminnensis*, compared to 22 for *H. magnivora* (Table 2).

Lack of recombination and asexual reproduction

By cloning and sequencing the PCR products of alpha-tub, betatub and hsp70, we were able to assign haplotypes to a few isolates (2–3) from each species. As no

Table 3 Polymorphism and recombination parameters estimated from 129 SNPs of 16 *Hamiltosporidium* genes (alignment on Table S2)

	Hamiltosporidium tvaerminnensis	*Hamiltosporidium magnivora*
Number of sequences	40	10
Number of haplotypes	7	10
Number of segregating sites – s	18	90
Average number of nucleotide differences – k	7.87	35.82
Nucleotide diversity – π	0.06	0.28
Tajima's D	2.81**	0.62^{NS}
Minimum number of recombination events – Rm	2	22
Average observed heterozygosity – H_O (SD)	0.80 (0.37)	0.36 (0.17)
Average expected heterozygosity – H_E (SD)	0.44 (0.17)	0.40 (0.14)
Fixation coefficient – F	-0.83**	0.04^{NS}

NS, not significant.
**$P < 0.01$

more than two haplotypes per isolate were found in both species, we assured that heterozygosity had been correctly inferred from the raw data, and concluded that both parasites occur in a diploid state within the *Daphnia* host. Cloning PCR products allowed us to rule out mixed infections or artefacts as a cause for sequencing double peaks. SNPs of *H. tvaerminnensis* always showed the same phase, suggesting lack of recombination. The diploid status of *Hamiltosporidium* spores is further supported by flow cytometry experiments, which consistently showed a single peak in the distribution of DAPI amounts per spore, regardless of host age (see Fig. S2 for details). This suggests that all spores in the *Daphnia* host have the same ploidy, that no reductional (meiotic) divisions are occurring, and – in the light of our SNP data showing heterozygosity – that spores are diploid.

Sex-related genes should suffer decay in truly asexual species (Normark *et al*. 2003). The *H. tvaerminnensis* genome draft (Corradi *et al*. 2009) does contain some elements of a MAT-type locus in separate contigs, but all our attempts to verify conserved synteny of these genes using PCR failed. Moreover, Spo11 (necessary to break the DNA before meiotic recombination), Hop2 (aids in the synapsis between chromosomes), Rec8 (involved in sister chromatid cohesion) and Rad21 (involved in repair of double strand breaks) have not been found in the *H. tvaerminnensis* genome draft (K. Haag, unpublished). These results have to be taken with caution, however, because the available draft is not complete.

The phase of eight alphatub, eight betatub and 18 hsp70 SNPs was estimated in our sample of 25 *Hamiltosporidium* isolates, by including the sequences derived from cloning experiments as known haplotypes. This allowed us to calculate the recombination parameter ρ for each gene (Table 4). Because the algorithm was run over 10 000 iterations, a distribution was achieved in which the median represents an estimate of ρ. In every case, recombination was several orders of magnitude larger in *H. magnivora* than in *H. tvaerminnensis* ($P < 0.001$).

Alignments containing the phased alleles for each locus were used to build maximum-likelihood gene trees (Fig. 2). Figure 2 shows that, excepting isolate FHS 1-8-1, only two alleles are found for each of the three selected markers in the *H. tvaerminnensis* sample ($n = 20$), in contrast with *H. magnivora*, that shows 10 alleles at each locus in a much smaller sample ($n = 5$). Pairwise average genetic distances were also calculated for each locus within each individual and compared using a Wilcoxon rank-sum test to verify significant differences in ASD between *H. tvaerminnensis* and *H. magnivora*. On average, within-individual haplotype distances in *H. tvaerminnensis* (0.004, 0.003 and 0.009 for alphatub, betatub and hsp70, respectively) were larger than in *H. magnivora* (0.001, 0.002 and 0.006). Significantly, larger ASD was confirmed for alphatub and hsp70 alleles within the *H. tvaerminnensis* sample ($P < 0.0001$; see Fig. 2) as compared with *H. magnivora*.

Discussion

Genetic markers and modes of reproduction in Microsporidia

Our study provides the first population genetic evidence on mode of reproduction in the phylum Microspora, based on an array of SNPs within 16 single-copy genes. Throughout its entire geographic range across the Skerry Islands of the Baltic Sea, *H. tvaerminnensis* shows a striking genetic homogeneity, that is, the presence of a repeated genotype in almost all sampled isolates. There is virtually fixed heterozygosity ($H_O = 0.80$), and a strongly negative F (−0.83), that we interpret as a consequence of an asexual mode of reproduction. For each of the three markers that have been chosen for recombination analyses (alphatub, betatub and hsp70), we found two major haplotypes in fixed heterozygosity throughout the entire Fennoscandian region. Our recombination estimates for these markers in *H. tvaerminnensis* did not differ significantly from zero. The significant and positive value of Tajima's D in *H. tvaerminnensis* also indicates the presence of two divergent haplotypes maintained in heterozygosity (and thus at equal frequencies) within the clonal population. Although both

Table 4 Recombination parameter estimates obtained from a subsample of 34 SNPs (shown in Fig. 1) and phased with the Phase software (SD = standard deviation)

	Alpha-tubulin		Beta-tubulin		Hsp70	
	Hamiltosporidium tvaerminnensis	*Hamiltosporidium magnivora*	*Hamiltosporidium tvaerminnensis*	*Hamiltosporidium magnivora*	*Hamiltosporidium tvaerminnensis*	*Hamiltosporidium magnivora*
Median	15.00E-05	0.043	26.90E-05	0.029	16.10E-05	0.091
Mean	6.06E-05	45.562	13.92E-05	31.069	3.81E-05	6.105
SD	23.03E-05	136.561	60.62E-05	109.842	12.27E-05	57.653

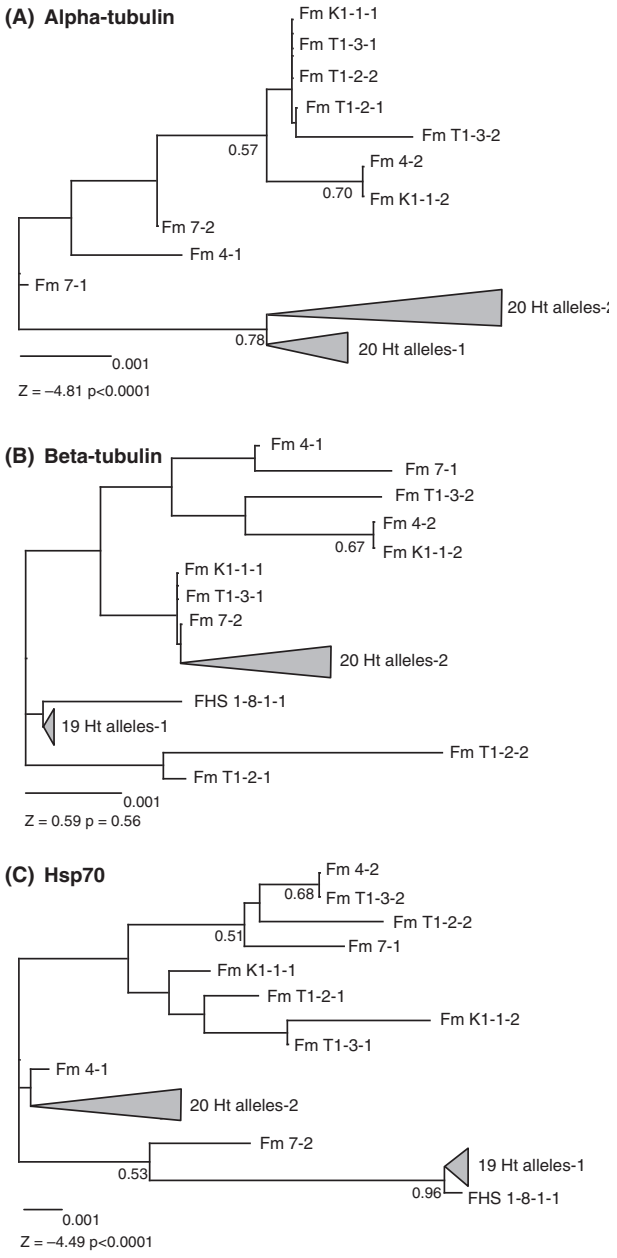

Fig. 2 Maximum-likelihood phylogenies of *Hamiltosporidium tvaerminnensis* (Ht) and *Hamiltosporidium magnivora* (Fm) alpha-tubulin, beta-tubulin and hsp70 haplotypes (alleles). Each individual allele is labelled with the corresponding isolate code (see Table 1) followed by number 1 or 2. As most *H. tvaerminnensis* isolates shared identical alleles, branches leading to them are shown in grey and labelled as Ht. Branches showing bootstrap support higher than 0.50 are indicated. Below each tree is shown the relative scale of branch lengths (genetic change), and the result of the Wilcoxon rank-sum test of ASD differences between *H. tvaerminnensis* and *H. magnivora*.

an excess of heterozygotes and a positive Tajima's *D* could in theory result from balancing selection, we have no reason to assume selection, since all SNPs analysed in our study are synonymous substitutions. Combined, these data argue for an obligately asexual status of *H. tvaerminnensis*. Our sampling strategy did not represent the deeper hierarchical levels of its genetic structure, which would be ideal for accurately estimating population parameters, and identifying putatively hidden structure (Halkett *et al.* 2005), but we opted for a more intensive survey of the potentially informative markers within the *Hamiltosporidium* genome. Data on microsporidian population genetic structure and recombination patterns are virtually nonexistent due to the absence of such markers. Most data available are based on multicopy rDNA sequences, which are known for their lack of concerted evolution in microsporidia (Tay *et al.* 2005; O'Mahony *et al.* 2007). PCR-based studies do not allow discriminating variation between alleles and different rDNA gene copies. Moreover, these genes are often involved in nonhomologous recombination and gene conversion, mechanisms that do not necessarily imply meiotic divisions (Liao *et al.* 1997). We think that the finding of repeated genotypes of *H. tvaerminnensis* throughout a wide distribution range in isolates that have been sampled over a period of five years strengthens the argument for clonality. Our relatively small sample of *H. magnivora* did not allow us to reliably assess its reproduction mode, but the fact that five isolates within a small geographic region show both high recombination rates and random assortment of SNPs strongly supports its sexual status. We are currently working on a larger set of markers and samples to verify the extent of sexual reproduction in natural *H. magnivora* populations.

Cytological observations, life cycles and reproduction modes

We suggest that both *H. tvaerminnensis* and *H. magnivora* are diploid within the *Daphnia* host, and that *Daphnia* does not work as a final host in the *Hamiltosporidium* life cycle, since no haploid spores have been found. Our results are compatible with our previous observations on the ultrastructure of *H. tvaerminnensis* sporogony in *D. magna*, that did not reveal any evidence of a meiotic process (Haag *et al.* 2011). Meiosis in microsporidia has been described as unusual, due to the particular arrangement of chromosomes during synapsis (Hazard & Brookbank 1984; Canning 1988). Nevertheless, it is unlikely that *Hamiltosporidium* cells undergoing meiotic divisions would have been lost during the spore purification process, since microsporidian meiospores are most often involved in horizontal transmission – known as environmental spores – and therefore protected by a thickened cell wall (Andreadis 2007). Furthermore, the spore populations that were used for DAPI quantification showed a very homogeneous distribution of sizes

and shapes in the flow cytometry analyses (data not shown). Although the life cycle of *H. magnivora* is not yet fully understood, we know that the species is vertically transmitted, and horizontal transmission was suggested to require a second (yet unknown) host (Mangin *et al.* 1995). *H. tvaerminnensis*, on the other hand, has a direct life cycle, being transmitted both vertically and horizontally from *Daphnia* to *Daphnia* (Vizoso & Ebert 2004). Thus, we suggest that colonization of the newly formed rock–pool habitat on the Skerry Islands, where the second host might have been absent, became possible only due to the *Daphnia* to *Daphnia* horizontal transmission. As we have no evidence of reductional divisions in the *Daphnia* host for either parasite species, we speculate that the sexual (or parasexual) process, and recombination, of *H. magnivora* occurs in the unknown second host (our hypothesis on the *Hamiltosporidium* cell cycle based on the flow cytometry experiments is depicted in Fig. S2). Unfortunately, knowledge of microsporidian life cycles is mostly incomplete. It is not known whether sexuality tends to be intimately associated with an indirect life cycle, as it is for the Amblyosporidae (Andreadis 2007). Nothing is known about the fate of meiospores of some species, such as *Vairimorpha necatrix* and *Theloania* spp, but it is speculated that they need an intermediate host for horizontal transmission as well (Ironside 2007). Traditionally, taxonomy of microsporidia was based on spore morphology and development within particular host species. The advent of molecular taxonomy led to reclassifications of several species, and it would not be surprising if some microsporidian species originally described in separate hosts in reality correspond to distinct life cycle stages of the same species. For example, *Pleistophora typicalis*, that parasitizes the fish *Myoxocephalus scorpius*, and *Pleistophora mulleri* that lives in the crustacean *Gammarus duebeni*, share high similarity in Rpb1 and SSU rDNA sequences, indicating that they could represent distinct developmental stages of the same species in different hosts (Ironside *et al.* 2008). Integration of field studies on a broader range of potential host species within ecosystems, and the population genetic analyses of their microsporidian parasites will certainly help filling up the gaps in our knowledge of microsporidian life cycles.

Lack of recombination, allele sequence divergence and local adaptation

Four genes required for meiotic recombination were not found in the *H. tvaerminnensis* genome draft. An intensive search for genes of various metabolic pathways revealed that hardly any of the expected genes are missing (Corradi *et al.* 2009), making the lack of these four genes related to the sexual cycle a prominent exception. We aim to fully assemble the *H. tvaerminnensis* genome in the near future and reliably assess this question. Consistent with our reasoning, a few genes involved with meiotic recombination were found to be absent from other microsporidia that lack evidence of a sexual cycle (Lee *et al.* 2010b). Lack of recombination promotes divergence between alleles within loci (ASD) due to the independent accumulation of mutations over time in homologous chromosomes (Birky 1996), but because this is a slow process, ASD is normally expected in ancient asexuals (Judson & Normark 1996). There is one case study on ASD in anciently asexual mycorrhizal fungi (Pawlowska & Taylor 2004), but no evidences of ASD in other fungal or protozoan pathogens known for being mainly clonal. *Giardia*, a protozoan parasite regarded as ancient asexual, seems to show enough recombination to prevent ASD, either due to genetic exchange between the two nuclei of a single cyst (Poxleitner *et al.* 2008) or to events of sexual reproduction with outcrossing (Cooper *et al.* 2007). Nevertheless, the occurrence of a parasexual cycle, that does not involve meiosis, cannot be ruled out, and the rates of recombination within populations are not known (Birky 2010). One difficulty in assessing sexual reproduction using population genetic tools in this system is dealing with the problem of multiple infections, which cannot be distinguished from heterozygosity. In our *Hamiltosporidum* sample, multiple infections have been ruled out by carefully inspecting the sequencing chromatograms for double peaks of similar heights, and a preliminary analysis of several microsatellite markers (not shown) confirms that all isolates analysed in the present study indeed constitute single infections.

We found evidences for significant ASD in alpha-tubulin and hsp70 genes of *H. tvaerminnensis*. This is rather surprising, since *H. tvaerminnensis* probably invaded the Skerry Islands no longer than 10 000 years ago, after they became ice-free. Consistent with this, only six of the 129 SNPs analysed in our study were polymorphic in *H. tvaerminnensis*, hinting that a single asexual genotype became epidemic and that these polymorphisms resulted from mutations that occurred after the colonization event. In contrast, the number of fixed heterozygous sites is twice as large (12; see Table 2). Phylogeographic studies showed that *D. magna* mitochondrial haplotypes from the Skerry Island metapopulation system are very closely related (De Gelas & De Meester 2005), and it is also known that rates of migration across rock–pools are large (Ebert *et al.* 2002). Thus, it is very likely that asexuality preceded the arrival of *H. tvaerminnensis* in the Skerry Islands and that the asexual clone was allowed to quickly expand its range along with its host. The high stability of the

H. tvaerminnensis–D. magna system in Fennoscandia seems to be unique amongst other host–pathogen systems that have been shown to involve clonal epidemic genotypes. The population genetic structure of the wheat leaf rust (*P. triticina*) shows significantly higher than expected heterozygosities, with alleles at neutral loci being strongly associated with virulence genes, suggesting host selection (Goyeau *et al.* 2007). Strong selection exerted by the host plant combined with large-scale migration in such plant–pathogen systems generates a periodic turnover of the fungus genotypic composition, because established genotypes are recurrently replaced by invading pathotypes (Hovmøller *et al.* 2002).

Our results seem to contradict an earlier finding that *H. tvaerminnensis* is locally adapted to *D. magna* in the rock–pool metapopulation system (Altermatt *et al.* 2007). In a common garden competition experiment, it was found that *H. tvaerminnensis* reduced the fitness of its local hosts relatively more than the fitness of allopatric host genotypes, originated from a different rock–pool (Altermatt *et al.* 2007). An explanation for the evidence of local adaptation in face of low levels of genetic variation is that local adaptation of the parasite would have epigenetic causes. Another, not mutually exclusive possibility is that *H. tvaerminnensis* harbours a few fast evolving, or polymorphic genes involved in parasite-host interactions. Within our study, the spore wall protein-encoding gene (SWP25) was the most variable within the *H. tvaerminnensis* sample, showing two polymorphic sites (Table 2), that is, two concomitant transversions, and loss of heterozygosity, in five *H. tvaerminnensis* isolates (see Table S2). As these two sites are separated by 444 bp in the SWP gene and as they have changed concomitantly in all five isolates, we suspect that loss of hetrozygosity might have resulted from gene conversion. Interestingly, there is evidence that spore wall proteins are involved in host–parasite interactions in microsporidia. Microsporidian parasites attach to host cells via glycosaminoglycans, and different spore wall proteins contain heparin motifs that were shown to bind glycosaminoglycans, besides being required for attachment (Southern *et al.* 2007). The *Hamiltosporidium* SWP25 gene is homologous to a highly expressed gene of *Nosema bombycis* (HSWP2; GenBank accession no. EF683102), which encodes a secreted spore wall protein that contains one heparin motif (Wu *et al.* 2008). The *N. bombycis* SWP25 protein is localized at the endospore, but does not seem to be directly involved in the attachment process (Wu *et al.* 2009). Protein sequences highly similar to the *H. tvarminnensis* and *N. bombycis* SWP25 were deduced from the genomes of other microsporidians, such as *E. cuniculi* and *Nosema ceranae*, but their function is unknown.

Nonrandom mitotic recombination or aneuploidy was found across the genome of *Batrachochytrium dendrobatidis*, a chytridiomycete parasitic fungus that is causing global declines in frog populations (James *et al.* 2009). As demonstrated by nearly fixed heterozygosity, the rapid population expansions in *B. dendrobatidis* are associated with clonal reproduction (Morehouse *et al.* 2003). The emerging pathogen shows larger decay in heterozygosity on its largest chromosome (James *et al.* 2009). Similarly, selective loss of chromosome 5 was found in *C. albicans* strains evolving in sorbose-containing media (Janbon *et al.* 1998). Mitotic recombination seems to be an efficient mechanism for exposing recessive adaptive alleles and increasing their rate of fixation in obligately asexual pathogens (Schoustra *et al.* 2007; Bougnoux *et al.* 2008; Sun & Xu 2009). Loss of heterozygosity has also been implicated in attenuation of virulence and acquisition of drug resistance (Chen *et al.* 2004; Selmecki *et al.* 2006; Wu *et al.* 2007). We are currently trying to disentangle the roles of genetic and epigenetic factors that in adaptation of *H. tvaerminnensis* to *D. magna*.

Conclusion and prospects

We have shown, using SNPs, that two closely related microsporidian species differing in their modes of transmission have different reproduction modes. Based on three lines of evidence, (i) lack of recombination, (ii) fixed heterozygosity and (iii) ASD, we suggest here that *H. tvaerminnensis* is asexual, whereas its sister species *H. magnivora* is sexual. *H. tvaerminnensis* is the first example of an asexual parasite that, besides several other free-living asexual species (see Kearney 2005), succeeded in colonizing an empty habitat after the last glaciation period. Asexuality is linked to the spread in a newly founded habitat (the rock–pool habitat of the Baltic Sea arose only after de-glaciation) and to a direct life cycle, but most probably originated before *H. tvaerminnensis* arrived in the Skerry Islands. A general theory of parasite–host coevolution still demands a better understanding of the conditions under which recombination is beneficial or not in parasites. The loss of sex seems to have been a recurrent event in the history of microsporidia (Ironside 2007), but its causes and the conditions under which it occurred might have been different for different species. With new examples emerging, it may be possible to use comparative approaches to trace the factors causing the loss of sexual reproduction in microsporidians. The availability of genomic data opens new avenues for population genetic studies that, coupled with a broader and more detailed description of microsporidian life histories and epidemiology may generate a comprehensive view on the evolution of mode of reproduction in this Phylum.

Acknowledgements

Thanks to Dita Vizoso and Thomas Zumbrunn for their help with the illustrations and to David Duneau for his help with sorting spores. Special thanks to Jason Andras, as well as the anonymous reviewers, for their critical reading of our manuscript. This study was supported by the Swiss National Science Foundation.

References

Altermatt F, Hottinger J, Ebert D (2007) Parasites promote host gene flow in a metapopulation. *Evolutionary Ecology*, **21**, 561–575.

Andreadis TG (2007) Microsporidian parasites of mosquitoes. *Journal of the American Mosquito Control Association*, **23**, 3–29.

den Bakker HC, VanKuren NW, Morton JB, Pawlowska TE (2010) Clonality and recombination in the life history of an asexual arbuscular mycorrhizal fungus. *Molecular Biology and Evolution*, **27**, 2474–2486.

Balloux F, Lehmann L, De Meeûs T (2003) The population genetics of clonal and partially clonal diploids. *Genetics*, **164**, 1635–1644.

Barrès B, Dutech C, Andrieux A, Halkett F, Frey P (2012) Exploring the role of asexual multiplication in poplar rust epidemics: impact on diversity and genetic structure. *Molecular Ecology*, **21**, 4996–5008.

Barton NH (2010) Mutation and the evolution of recombination. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **365**, 1281–1294.

Barton NH, Charlesworth B (1998) Why sex and recombination? *Science*, **281**, 1986–1990.

Birky CW (1996) Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. *Genetics*, **144**, 427–437.

Birky CW (2010) *Giardia* sex? Yes, but how and how much? *Trends in Parasitology*, **26**, 70–74.

Bougnoux M-E, Pujol C, Diogo D, Bouchier C, Soll DR, d'Enfert C (2008) Mating is rare within as well as between clades of the human pathogen *Candida albicans*. *Fungal Genetics and Biology*, **45**, 221–231.

Canning EU (1988) Nuclear division and chromosome cycle in microsporidia. *Biosystems*, **21**, 333–340.

Canning EU, Curry A, Cheney S, Lafranchi-Tristem NJ, Haque MA (1999) *Vairimorpha imperfecta* n.sp., a microsporidian exhibiting an abortive octosporous sporogony in *Plutella xylostella* L. (Lepidoptera: Yponomeutidae). *Parasitology*, **119**, 273–286.

Ceplitis A (2003) Coalescence times and the Meselson effect in asexual eukaryotes. *Genetics Research*, **82**, 183–190.

Chen WJ, Barr AR (1995) Chromosomal evidence on the sporogony of *Amblyospora californica* (Microspora: Amblyosporidae) in *Culex tarsalis* (Diptera: Culicidae). *The Journal of Eukaryotic Microbiology*, **42**, 103–108.

Chen X, Magee BB, Dawson D, Magee PT, Kumamoto CA (2004) Chromosome 1 trisomy compromises the virulence of *Candida albicans*. *Molecular Microbiology*, **51**, 551–565.

Cooper MA, Adam RD, Worobey M, Sterling CR (2007) Population genetics provides evidence for recombination in *Giardia*. *Current Biology*, **17**, 1984–1988.

Corradi N, Haag KL, Pombert J-F, Ebert D, Keeling PJ (2009) Draft genome sequence of the *Daphnia* pathogen *Octosporea bayeri*: insights into the gene content of a large microsporidian genome and a model for host-parasite interactions. *Genome Biology*, **10**, R106.

Crawford DC, Bhangale T, Li N *et al.* (2004) Evidence for substantial fine-scale variation in recombination rates across the human genome. *Nature Genetics*, **36**, 700–706.

De Gelas K, De Meester L (2005) Phylogeography of *Daphnia magna* in Europe. *Molecular Ecology*, **14**, 753–764.

Decaestecker E, Declerck S, De Meester L, Ebert D (2005) Ecological implications of parasites in natural *Daphnia* populations. *Oecologia*, **144**, 382–390.

Ebert D, Hottinger JW, Pajunen VI (2001) Temporal and spatial dynamics of parasite richness in a *Daphnia* metapopulation. *Ecology*, **82**, 3417–3434.

Ebert D, Haag C, Kirkpatrick M *et al.* (2002) A selective advantage to immigrant genes in a *Daphnia* metapopulation. *Science*, **295**, 485–488.

Enjalbert J, Duan X, Leconte M, Hovmller MS, De Vallavieille-Pope C (2005) Genetic evidence of local adaptation of wheat yellow rust (*Puccinia striiformis* f. sp. *tritici*) within France. *Molecular Ecology*, **14**, 2065–2073.

Excoffier L, Laval G, Schneider S (2007) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics*, **2005**, 47–50.

Flegel TW, Pasharawipas T (1995) A proposal for typical eukaryotic meiosis in microsporidians. *Canadian Journal of Microbiology*, **41**, 1–11.

Gatehouse (née Edmonds) HS, Malone LA (1998) The ribosomal RNA gene region of *Nosema apis* (Microspora): DNA sequence for small and large subunit rRNA genes and evidence of a large tandem repeat unit size. *Journal of Invertebrate Pathology*, **71**, 97–105.

Goyeau H, Halkett F, Zapater M-F, Carlier J, Lannou C (2007) Clonality and host selection in the wheat pathogenic fungus *Puccinia triticina*. *Fungal Genetics and Biology*, **44**, 474–483.

Haag KL, Larsson JIR, Refardt D, Ebert D (2011) Cytological and molecular description of *Hamiltosporidium tvaerminnensis* gen. et sp. nov., a microsporidian parasite of *Daphnia magna*, and establishment of *Hamiltosporidium magnivora* comb. nov. *Parasitology*, **138**, 447–462.

Halkett F, Simon J-C, Balloux F (2005) Tackling the population genetics of clonal and partially clonal organisms. *Trends in Ecology & Evolution*, **20**, 194–201.

Hazard EI, Brookbank JW (1984) Karyogamy and meiosis in an *Amblyospora* sp. (Microspora) in the mosquito *Culex salinarius*. *Journal of Invertebrate Pathology*, **44**, 3–11.

Hazard EI, Fukuda T, Becnel JJ (1985) Gametogenesis and plasmogamy in certain species of Microspora. *Journal of Invertebrate Pathology*, **46**, 63–69.

Hedrick PW (2011) *Genetics of Populations*. Jones & Bartlett Publishers, Sudbury, Massachusetts.

Heitman J (2006) Sexual reproduction and the evolution of microbial pathogens. *Current Biology*, **16**, R711–R725.

Heitman J (2010) Evolution of eukaryotic microbial pathogens via covert sexual reproduction. *Cell Host & Microbe*, **8**, 86–99.

Hovmøller MS, Justesen AF, Brown JKM (2002) Clonality and long-distance migration of *Puccinia striiformis* f.sp. *tritici* in north-west Europe. *Plant Pathology*, **51**, 24–32.

Hurst R (2000) Transitions in the evolution of meiosis. *Journal of Evolutionary Biology*, **13**, 466–479.

Hurst LD, Hamilton WD, Ladle RJ (1992) Covert sex. *Trends in Ecology & Evolution*, **7**, 144–145.

Idnurm A, Walton FJ, Floyd A, Heitman J (2008) Identification of the sex genes in an early diverged fungus. *Nature*, **451**, 193–196.

Ironside JE (2007) Multiple losses of sex within a single genus of Microsporidia. *BMC Evolutionary Biology*, **7**, 48.

Ironside JE, Wilkinson TJ, Rock J (2008) Distribution and host range of the microsporidian *Pleistophora mulleri*. *Journal of Eukaryotic Microbiology*, **55**, 355–362.

James TY, Kauff F, Schoch CL *et al.* (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature*, **443**, 818–822.

James TY, Litvintseva AP, Vilgalys R *et al.* (2009) Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. *PLoS Pathogens*, **5**, e1000458.

Janbon G, Sherman F, Rustchenko E (1998) Monosomy of a specific chromosome determines l-sorbose utilization: a novel regulatory mechanism in *Candida albicans*. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 5150–5155.

Judson OP, Normark BB (1996) Ancient asexual scandals. *Trends in Ecology & Evolution*, **11**, 41–46.

Kearney M (2005) Hybridization, glaciation and geographical parthenogenesis. *Trends in Ecology & Evolution*, **20**, 495–502.

Koffi M, Meeûs TD, Bucheton B *et al.* (2009) Population genetics of *Trypanosoma brucei gambiense*, the agent of sleeping sickness in Western Africa. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 209–214.

Kolmer JA, Hanzalova A, Goyeau H, Bayles R, Morgounov A (2012) Genetic differentiation of the wheat leaf rust fungus *Puccinia triticina* in Europe. *Plant Pathology*, doi: 10.1111/j.1365-3059.2012.02626.x.

Kuhn G, Hijri M, Sanders IR (2001) Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. *Nature*, **414**, 745–748.

Lass S, Ebert D (2006) Apparent seasonality of parasite dynamics: analysis of cyclic prevalence patterns. *Proceedings of the Royal Society B: Biological Sciences*, **273**, 199–206.

Lee SC, Corradi N, Byrnes III EJ *et al.* (2008) Microsporidia evolved from ancestral sexual Fungi. *Current Biology*, **18**, 1675–1679.

Lee SC, Corradi N, Doan S *et al.* (2010a) Evolution of the sex-related locus and genomic features shared in Microsporidia and Fungi. *PLoS ONE*, **5**, e10539.

Lee SC, Ni M, Li W, Shertz C, Heitman J (2010b) The evolution of sex: a perspective from the fungal kingdom. *Microbiology and Molecular Biology Reviews*, **74**, 298–340.

Li N, Stephens M (2003) Modeling linkage disequilibrium and identifying recombination hotspots using single-nucleotide polymorphism data. *Genetics*, **165**, 2213–2233.

Li W, Cama V, Feng Y *et al.* (2012) Population genetic analysis of *Enterocytozoon bieneusi* in humans. *International Journal for Parasitology*, **42**, 287–293.

Liao D, Pavelitz T, Kidd JR, Kidd KK, Weiner AM (1997) Concerted evolution of the tandemly repeated genes encoding human U2 snRNA (the RNU2 locus) involves rapid intrachromosomal homogenization and rare interchromosomal gene conversion. *The EMBO Journal*, **16**, 588–598.

Librado P, Rozas J (2009) DnaSP V5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, **25**, 1451–1452.

Mangin KL, Lipsitch M, Ebert D (1995) Virulence and transmission modes of two microsporidia in *Daphnia magna*. *Parasitology*, **111**, 133–142.

Mathis A (2000) Microsporidia: emerging advances in understanding the basic biology of these unique organisms. *International Journal for Parasitology*, **30**, 795–804.

de Meeûs T, Balloux F (2004) Clonal reproduction and linkage disequilibrium in diploids: a simulation study. *Infection, Genetics and Evolution*, **4**, 345–351.

Morehouse EA, James TY, Ganley ARD *et al.* (2003) Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. *Molecular Ecology*, **12**, 395–403.

Nébavi F, Ayala FJ, Renaud F *et al.* (2006) Clonal population structure and genetic diversity of *Candida albicans* in AIDS patients from Abidjan (Côte d'Ivoire). *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 3663–3668.

Normark BB, Judson OP, Moran NA (2003) Genomic signatures of ancient asexual lineages. *Biological Journal of the Linnean Society*, **79**, 69–84.

O'Mahony EM, Tay WT, Paxton RJ (2007) Multiple rRNA variants in a single spore of the microsporidian *Nosema bombi*. *Journal of Eukaryotic Microbiology*, **54**, 103–109.

Pawlowska TE, Taylor JW (2004) Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. *Nature*, **427**, 733–737.

Poxleitner MK, Carpenter ML, Mancuso JJ *et al.* (2008) Evidence for karyogamy and exchange of genetic material in the binucleate intestinal parasite *Giardia intestinalis*. *Science*, **319**, 1530–1533.

Ramesh MA, Malik S-B, Logsdon Jr JM (2005) A phylogenomic inventory of meiotic genes: evidence for sex in *Giardia* and an early eukaryotic origin of meiosis. *Current Biology*, **15**, 185–191.

Rich SM, Hudson RR, Ayala FJ (1997) *Plasmodium falciparum* antigenic diversity: evidence of clonal population structure. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 13040–13045.

Schoustra SE, Debets AJM, Slakhorst M, Hoekstra RF (2007) Mitotic recombination accelerates adaptation in the fungus *Aspergillus nidulans*. *PLoS Genetics*, **3**, e68.

Selmecki A, Forche A, Berman J (2006) Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science*, **313**, 367–370.

Sibley LD, Ajioka JW (2008) Population structure of *Toxoplasma gondii*: clonal expansion driven by infrequent recombination and selective sweeps. *Annual Review of Microbiology*, **62**, 329–351.

Southern TR, Jolly CE, Lester ME, Hayman JR (2007) EnP1, a microsporidian spore wall protein that enables spores to adhere to and infect host cells in vitro. *Eukaryotic Cell*, **6**, 1354–1362.

Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics*, **68**, 978–989.

Sun S, Xu J (2009) Chromosomal rearrangements between serotype A and D strains in *Cryptococcus neoformans*. *PLoS ONE*, **4**, e5524.

Sweeney AW, Becnel JJ (1991) Potential of microsporidia for the biological control of mosquitoes. *Parasitology Today*, **7**, 217–220.

Sweeney AW, Graham MF, Hazard EI (1988) Life cycle of *Amblyospora dyxenoides* sp. nov. in the mosquito *Culex annulirostris* and the copepod *Mesocyclops albicans*. *Journal of Invertebrate Pathology*, **51**, 46–57.

Sweeney AW, Doggett SL, Piper RG (1990) Life cycle of *Amblyospora indicola* (Microspora: Amblyosporidae), a parasite of the mosquito *Culex sitiens* and of *Apocyclops* sp. Copepods. *Journal of Invertebrate Pathology*, **55**, 428–434.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, **28**, 2731–2739.

Tay WT, O'Mahony EM, Paxton RJ (2005) Complete rRNA gene sequences reveal that the microsporidium *Nosema bombi* infects diverse bumblebee (*Bombus* spp.) hosts and contains multiple polymorphic sites. *Journal of Eukaryotic Microbiology*, **52**, 505–513.

Tibayrenc M, Ayala FJ (1991) Towards a population genetics of microorganisms: the clonal theory of parasitic protozoa. *Parasitology Today*, **7**, 228–232.

Tibayrenc M, Kjellberg F, Ayala FJ (1990) A clonal theory of parasitic protozoa: the population structures of *Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas,* and *Trypanosoma* and their medical and taxonomical consequences. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 2414–2418.

Vizoso DB, Ebert D (2004) Within-host dynamics of a microsporidium with horizontal and vertical transmission: *Octosporea bayeri* in *Daphnia magna*. *Parasitology*, **128**, 31–38.

Wu W, Lockhart SR, Pujol C, Srikantha T, Soll DR (2007) Heterozygosity of genes on the sex chromosome regulates *Candida albicans* virulence. *Molecular Microbiology*, **64**, 1587–1604.

Wu Z, Li Y, Pan G *et al.* (2008) Proteomic analysis of spore wall proteins and identification of two spore wall proteins from *Nosema bombycis* (Microsporidia). *Proteomics*, **8**, 2447–2461.

Wu Z, Li Y, Pan G, Zhou Z, Xiang Z (2009) SWP25, a novel protein associated with the *Nosema bombycis* endospore. *Journal of Eukaryotic Microbiology*, **56**, 113–118.

Xiao L, Li L, Visvesvara GS, Moura H, Didier ES, Lal AA (2001) Genotyping *Encephalitozoon cuniculi* by multilocus analyses of genes with repetitive sequences. *Journal of Clinical Microbiology*, **39**, 2248–2253.

K.L.H. is an evolutionary biologist interested in molecular epidemiology and evolutionary genetics of infectious diseases. E.T. is a technician interested in the diversity of cell types and their cycles. D.E. is an ecologist interested in host-parasite coevolution.

Data accessibility

The genetic markers searched for SNPs in the present study came from a whole-genome shotgun (WGS) database of the *H. tvaerminnensis* isolate OER-3-3 deposited at NCBI with access ACSZ00000000. The supporting information contains: (i) a list of the contigs used for polymorphism analyses, with their respective annotation and primers (Table S1) and (ii) an alignment of the concatenated SNPs of all *Hamiltosporidium* isolates (Table S3).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Genetic markers, their corresponding contigs the *Hamiltosporidium tvaerminnensis* genome database, and their homologs *in Encephalitozoon cuniculi.*

Table S2 Alignment of the 129 unphased SNPs and five indels found in our sample.

Fig. S1 Forward and reverse sequencing reads showing heterozygous SNPs in different isolates and genes.

Fig. S2 Observations on the life cycle of *Hamiltosporidium tvaerminnensis* and *Hamiltosporidium magnivora.*