Widespread vertical transmission and associated host sex-ratio distortion within the eukaryotic phylum Microspora

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Vertical transmission (VT) and associated manipulation of host reproduction are widely reported among prokaryotic endosymbionts. Here, we present evidence for widespread use of VT and associated sex-ratio distortion in a eukaryotic phylum. The Microspora are an unusual and diverse group of eukaryotic parasites that infect all animal phyla. Following our initial description of a microsporidian that feminizes its crustacean host, we survey the diversity and distribution of VT within the Microspora. We find that vertically transmitted microsporidia are ubiquitous in the amphipod hosts sampled and that they are also diverse, with 11 species of microsporidia detected within 16 host species. We found that infections were more common in females than males, suggesting that host sex-ratio distortion occurs in five out of eight parasite species tested. Phylogenetic reconstruction demonstrates that VT occurs in all major lineages of the phylum Microspora and that sex-ratio distorters are found on multiple branches of the phylogenetic tree. We propose that VT is either an ancestral trait or evolves with peculiar frequency in this phylum. If the association observed here between VT and host sex-ratio distortion holds true across other host taxa, these eukaryotic parasites may join the bacterial endosymbionts in their importance as sex-ratio distorters.

Keywords: vertical transmission; sex-ratio distortion; Microsporidia

1. INTRODUCTION

The Microspora are an unusual and diverse group of eukaryotic parasites that infect all animal phyla from protists to humans (Wittner & Weiss 1999). Their unusual cellular characteristics, including possession of 70s ribosomes and an apparent lack of mitochondria, originally placed them as 'ancient' deep-branching eukaryotes (Vossbrink *et al.* 1987). However, the recent discovery of a relictual mitochondrial organelle (Katinka *et al.* 2001; Williams *et al.* 2002), together with revised phylogenetic analysis, confirms that they are in fact a highly derived sister group to the fungi (Hirt *et al.* 1999; Baldauf 2003).

Microsporidia have complex life cycles that may use both vertical and horizontal transmission (Dunn & Smith 2001). The role of vertical transmission (VT) in the life cycle varies (Dunn *et al.* 2001): in some cases it is described as supplementary to the main horizontal route; however, in others an obligate alternative cycle is seen, and sole VT has also been reported (Terry *et al.* 1999a). Among bacterial endosymbionts, VT is associated with manipulations of

host reproduction (Bandi *et al.* 2001). These organisms cause parthenogenesis (Stouthamer *et al.* 1993; Weeks *et al.* 2001), cytoplasmic incompatibility (Tram & Sullivan 2002), male killing (Hurst & Jiggins 2000) and feminization (Bouchon *et al.* 1998).

We have previously described a microsporidian parasite, *Nosema granulosis*, which appears to be solely vertically transmitted and feminizes its crustacean host (Terry *et al.* 1999a; Ironside *et al.* 2003a). In parallel with the bacterial endosymbionts, this parasite has efficient strategies for transovarial transmission and does not cause patent pathology (Terry *et al.* 1997, 1998, 1999b). The association between VT and reduced pathogenesis means that vertically transmitted parasites are cryptic and would not be detected by normal disease-associated screening methods (Dunn & Smith 2001). This raises the questions of how common VT is across the phylum Microspora and how frequently it has led to host reproductive manipulations. Although few specific studies have been completed, vertically transmitted microsporidia have been shown to cause sex-ratio distortion via late male killing in dipteran hosts (Andreadis & Hall 1979) and to cause feminization in the amphipod *Gammarus duebeni* (Terry *et al.* 1999a). Here, we complete an unbiased survey of amphipod hosts to
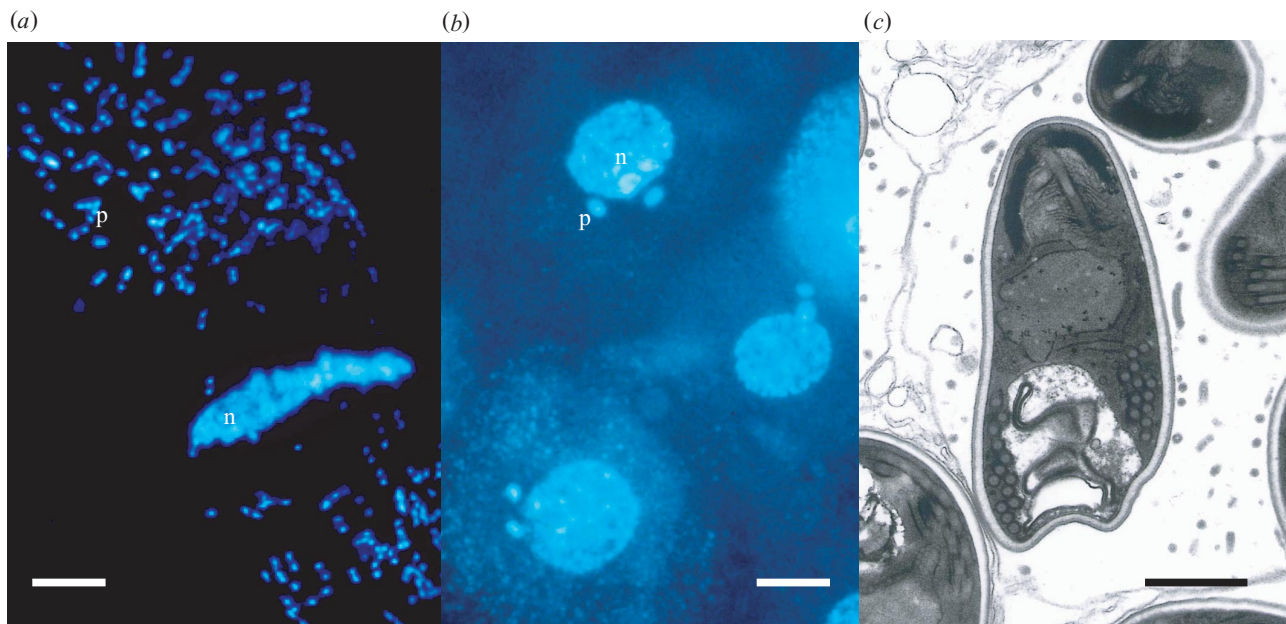
Figure 1. Microsporidian infection in amphipod hosts. (*a*) A *Gammarus duebeni* embryo infected with *Nosema granulosis*, stained with the DNA fluorescent dye DAPI showing the parasites (p) arranged in the perinuclear cytoplasm of the host nucleus (n). Scale bar, 5 μm. (*b*) A *G. duebeni* embryo infected with *Dictyocoela duebenum*, stained with the DNA fluorescent dye DAPI. Scale bar, 5 μm. (*c*) An electron micrograph of the spore stage of the novel microsporidian *D. muelleri* in the amphipod host *G. d. celticus*. Scale bar, 1 μm.

assess the diversity and distribution of vertically trans-mitted microsporidia and to test for host sex-ratio distor-tion. We further reconstruct the phylogeny of the Microspora to consider the origins of VT and sex-ratio dis-tortion traits.

2. MATERIAL AND METHODS

(a) *Screening*

Wild-caught amphipods were sampled from sites in northern Europe. *Gammarus duebeni duebeni* was sampled from populations on the Isle of Man (Hogg *et al.* 2002) and the Isle of Cumbrae (Ironside *et al.* 2003a) in the UK. *Gammarus duebeni celticus* was sampled from populations in Lough Neagh (MacNeil *et al.* 2003) and Downhill stream in Northern Ireland (Terry *et al.* 2003). The remaining species were from single populations in Averton Gif-ford, Devon (*G. chevreuxi*), Droitwich, Worcestershire (*G. tigri-nus*), Isle of Cumbrae, Scotland (*G. pulex*, *Orchestia cavimana*, *O. gammarellus*), Budle Bay, Northumberland (*Chaetogammarus stoerensis*, *G. zaddachi*), Anglesey, Wales (*G. locusta*) and Porta-ferry, Northern Ireland (*Echinogammarus marinus*) in the UK and from Poitiers (*E. berilloni*), Ouche river, Dijon (*G. roeseli*) and Isle d'Oleron (*Melita palmata*, *Microdeutopus gryllotalpa*, *Talitrus* sp., *Talorchestia deshayesii*) in France. Adult females were screened for vertically transmitted microsporidia via PCR of ovarian tissue using microsporidian-specific small subunit (SSU) ribosomal DNA (rDNA) primers (V1f, 530r, 530f, 580r; Weiss *et al.* 1994) as described previously (Terry *et al.* 1999a; Hogg *et al.* 2002). Additional primer sets (Terry *et al.* 2003) were employed where PCR products were faint, and negative samples were screened on a minimum of three occasions. The quality of DNA was checked using host Cytochrome C oxidase subunit I primers (Ironside *et al.* 2003a) and any negative samples were excluded from analysis. In addition to PCR screening, where possible, VT was confirmed by 4,6-diamidino-2-phenyl-indole (DAPI) staining of developing embryos obtained from females brooding eggs. As in previous studies there was 100% correspondence between positive or negative PCR diagnosis of ovarian tissue and the presence or absence of parasites in embryos (Hogg *et al.* 2002).

When run out on 1.5% agarose gels, variation was apparent in the size of PCR products. The full-length sequence was generated from each variant PCR product within each host population. Where sample size permitted, the sequence was generated from a minimum of five individuals in each case. PCR products were cleaned using Gel Extraction Kits (Qiagen) and sequenced at the University of Leeds or at the Natural History Museum, London. Approximately 1400 bp of SSU rDNA sequence was generated for each parasite isolate, and sequence representatives of the eight isolates were submitted to GenBank.

(b) *Sex-ratio distortion screen*

DNA was extracted from the gonads of at least 30 male and 30 female individuals and screened by PCR and sequencing. As above, the quality of DNA was checked using host COI primers (Ironside *et al.* 2003a), and any negative samples were excluded from analysis. Additional primer sets (Terry *et al.* 2003) were employed where PCR products were faint, and negative samples were screened on a minimum of three occasions. Parasite pre-valences in males and females were compared using Fisher's exact tests under the null hypothesis that the frequency of parasites will be the same in males and females.

(c) *Phylogenetic analyses*

Sequences were aligned by eye using MACCLADE, v. 4.0 (Maddi-son & Maddison 2001) removing all regions of ambiguity. Sites with single autapomorphic insertions were excluded to reduce branch lengths, and regions of inclusion tended to begin and end on single bases. Sequences were rooted against four species of Zygomycetes representing four orders, Mucorales, Ento-mophthorales, Zoopagales and Mortierellales. Full alignments are
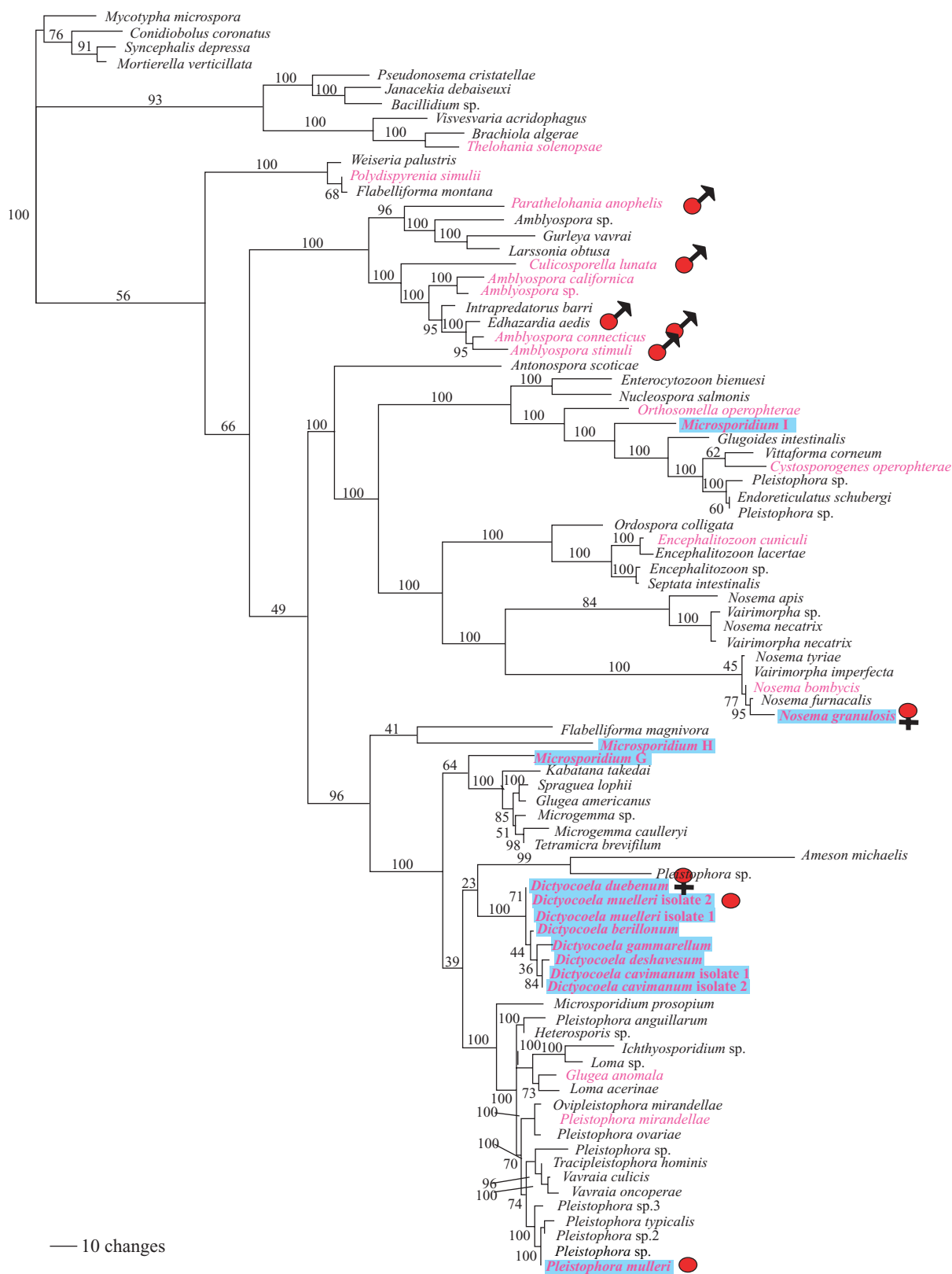
Figure 2. Microsporidian phylogenetic tree based on SSU rDNA of 83 ingroup and four outgroup taxa using Bayesian inference. Nodal support is given via posterior probabilities. VT has been observed in taxa throughout the phylum (in pink). Other taxa (in black) are unscreened; thus the presence or absence of the VT route is unknown. Taxa for which we have evidence of sex-ratio distortion (circles) occur in diverse areas of the tree: feminizing Microsporidia (female symbols) are found in two lineages and male killers (male symbols) in a third. Microsporidia infecting amphipods (highlighted in blue) are found in diverse branches of the tree. Trees generated by ME analysis (data not shown) gave similar topology and placement of the novel parasite sequences.

available from the EMBLALIGN database via SRS at http://srs.ebi.ac.uk, under the accession ALIGN_000626. A minimum evolution (ME) tree was estimated with PAUP* v. 4.0b10

(Swofford 2002). MODELTEST v. 3.06 was used to predict the best substitution model (general time reversible including estimates of invariant sites and among-site rate heterogeneity (Posada &

Table 1. Microsporidia detected from amphipod hosts.

(*N*, the number of individuals sampled within a host species; NI, the number of infected individuals within a host species. *Microsporidium* is a holding genus for unclassified microsporidian parasites.)

host species	*N*	parasites detected	NI	GenBank
Gammarus duebeni duebeni [a]	195	*Nosema granulosis*	70	AJ011833
		Dictyocoela duebenum	7	AF397404
		Dictyocoela berillonum	1	AJ438957
Gammarus duebeni celticus	121	*Nosema granulosis*	7	AJ011833
		Pleistophora mulleri	12	AJ438985
		Dictyocoela muelleri	3	AJ438955
Gammarus roeseli [a]	87	*Nosema granulosis*	13	AJ438956
		Dictyocoela muelleri	18	AJ438956
Chaetogammarus stoerensis [a]	1	*Nosema granulosis*	1	AJ011833
Gammarus pulex [a]	40	*Nosema granulosis*	1	AJ011833
		Dictyocoela duebenum	18	AF397404
		Microsporidium I	2	AJ438964
Gammarus tigrinus	60	*Dictyocoela duebenum*	10	AF397404
		Dictyocoela berillonum	2	AJ438957
Gammarus zaddachi [a]	59	*Nosema granulosis*	16	AJ011833
Gammarus locusta	15	*Dictyocoela duebenum*	3	AF397404
Echinogammarus berilloni [a]	193	*Dictyocoela duebenum*	53	AF397404
		Dictyocoela berillonum	14	AJ438957
Echinogammarus marinus	60	*Dictyocoela berillonum*	20	AJ438957
Melita palmata	60	*Dictyocoela berillonum*	7	AJ438957
Microdeutopus gryllotalpa	5	*Microsporidium* H	1	AJ438963
Talitrus sp.	68	*Dictyocoela cavimanum*	54	AJ438959
		Microsporidium H	1	AJ438963
Talorchestia deshayesii	60	*Dictyocoela deshayesum*	23	AJ438961
		Microsporidium H	1	AJ438963
Orchestia cavimana	52	*Dictyocoela cavimanum*	10	AJ438960
Orchestia gammarellus	60	*Dictyocoela gammarellum*	3	AJ438958
Gammarus chevreuxi [a]	41	*Microsporidium* G	7	AJ438962

[a] VT confirmed by screening of embryos.

Crandall 1998); GTR+I+G) for estimating a distance matrix used in the ME analysis. Nodal support was assessed by bootstrap resampling (1000 replicates). MRBAYES v. 3.0 (Huelsenbeck & Ronquist 2001) was used to infer a Bayesian analysis with a GTR+I+G substitution model over 500 000 generations, and nodal support was assessed by posterior probabilities estimated from the final 75% (3750) sampled trees.

3. RESULTS

We screened 16 species of amphipod Crustacea from field sites in northern Europe. Parasites were detected via PCR of parasite DNA from host gonadal tissue using microsporidian-specific SSU rDNA primers and by direct visualization in host embryos using fluorescence microscopy (figure 1*a*,*b*). Vertically transmitted microsporidia were detected in 100% of amphipod host species tested (table 1) suggesting that they are extremely widespread in this host group.

Analysis of parasite SSU rDNA and reconstruction of the phylogeny of the Microspora (figure 2) revealed that vertically transmitted microsporidia are very diverse. Eleven distinct microsporidian sequences were detected, of which nine were novel. Although parasites were found to coexist within host populations, there were no instances of dual infection in a single individual. One sequence corresponded to *N. granulosis*, a vertically transmitted parasite previously described from the ovarian tissue of *G. duebeni* but detected here in the subspecies *G. d. celticus* and in three further gammarid hosts.

A group of eight novel sequences fell into a discrete clade basal to the major lineage of microsporidia infecting fishes. On the basis of sequence homology and structure, we have designated six species, placing isolates within the same species where sequence homology falls within 1%. We propose that these parasites constitute a new genus, which we have provisionally designated *Dictyocoela*, based on the tubular network that fills the sporophorous vesicle containing developing parasite spores (figure 1*c*). The distribution of *Dictyocoela* species within the host differs from that of *N. granulosis* in that parasites are located in both ovarian tissue and adjacent muscle.

The remaining microsporidian sequences fell into discrete branches of the phylum Microspora. Two of these, *Pleistophora mulleri* and *Microsporidium* G, lay within a lineage that primarily contains virulent parasites of fishes, while *Microsporidium* H and *Microsporidium* I fell into discrete lineages.

To assess the extent to which VT is linked with sex-ratio distortion we used a PCR-based screen to compare parasite prevalence in gonadal tissue from individual male and female hosts from the field. A parasite that either feminizes or kills male offspring will be found in females more often than in males. A higher frequency of infection in females than in males is supportive of host sex-ratio distortion, although differential parasite survival in male and female hosts cannot be discounted. The test was applied to populations of 10 amphipod host species harbouring eight species of vertically transmitted microsporidia. Five of

Table 2. Sex-ratio distortion in vertically transmitted microsporidia.

parasite	amphipod host	frequency in females	frequency in males	p
Nosema granulosis	*Gammarus duebeni duebeni*	6/33	0/30	0.016*
	Gammarus roeseli	13/57	0/30	0.002**
Pleistophora mulleri	*Gammarus duebeni celticus*	10/30	2/30	0.011*
Dictyocoela duebenum	*Gammarus duebeni duebeni*	7/33	1/30	0.036*
	Gammarus tigrinus	8/30	2/30	0.039*
	Echinogammarus berilloni	33/96	20/97	0.024*
Dictyocoela cavimanum	*Orchestia cavimana*	8/22	2/30	0.009**
	Talitrus sp.	26/30	28/30	0.34 n.s.
Dictyocoela muelleri	*Gammarus roeseli*	17/57	1/57	0.000**
	Gammarus duebeni celticus	2/32	1/29	0.53 n.s.
Dictyocoela berillonum	*Echinogammarus berelloni*	7/96	7/97	0.6 n.s.
	Echinogammarus marinus	10/30	10/30	0.6 n.s.
	Gammarus duebeni duebeni	0/33	1/30	0.48 n.s.
	Gammarus tigrinus	1/30	1/30	0.75 n.s
	Melita palmata	4/30	3/30	0.5 n.s.
Dictyocoela deshayesum	*Talorchestia deshayesii*	9/24	14/28	0.27 n.s.
Dictyocoela gammarellum	*Orchestia gammarellus*	0/30	3/30	0.118 n.s.

*$p < 0.05$; **$p < 0.01$; n.s., not significant.

these parasite species showed a significant female bias in their distribution, indicating that they are sex-ratio distorters (table 2). *Nosema granulosis* and *D. duebenum* appear to distort sex ratio in all host species tested, while *D. cavimanum* and *D. muelleri* showed female-biased distributions in only one out of two hosts tested. We found no evidence for sex-ratio distortion by *D. berillonum*, *D. deshayesum* or *D. gammarellum*.

To consider the origins of VT and its association with sex-ratio distortion across the phylum we mapped the distributions of these traits onto the phylogenetic tree (figure 2). In addition to data from our systematic screen of amphipods, we incorporated data from the literature on all species for which VT or host sex-ratio distortion has been established. We find that VT is found in all lineages and that the ability of parasites to distort host sex ratio also occurs in diverse branches of the phylum.

4. DISCUSSION

The extent to which VT might be underestimated is revealed by our survey. Our screen of amphipod Crustacea revealed the presence of vertically transmitted microsporidia in 100% of host species. None of these parasites caused patent pathogenesis, demonstrating that vertically transmitted microsporidia are often cryptic and will be detected only by direct screening. The high incidence of infection and the species richness suggest that these parasites are of great importance to this host group. In comparison, species of the endosymbiotic bacteria *Wolbachia* have been estimated to infect up to 76% of insect species (Jeyaprakash & Hoy 2000; Jiggins *et al.* 2001; Shoemaker *et al.* 2002) and 35% of isopod crustaceans (Bouchon *et al.* 1998), whereas the Cytophaga-like organism associated with sex-ratio distortion of spider mites (Weeks *et al.* 2001) occurs in 7.2% of insect and mite species screened (Weeks *et al.* 2003).

The sequence data generated from our survey were used together with sequences on GenBank to reconstruct the phylogeny of the Microspora. The novel vertically transmitted parasites from amphipods were distributed among

diverse branches of the phylum. One of the species, *N. granulosis*, had previously been demonstrated to have a feminizing influence on host offspring (Terry *et al.* 1999a). This parasite falls in the genus *Nosema* within a clade containing the type species *N. bombycis*, which is responsible for pebrine disease in silkworms. This genus primarily contains parasites of Lepidoptera in which VT supplements the horizontal route. *Nosema granulosis* is the exception as it is the only species to infect non-lepidopteran hosts, to cause limited pathology and for which there is no evidence of horizontal transmission (Terry *et al.* 1997; Ironside *et al.* 2003a). A group of eight novel sequences fall into a discrete clade basal to the major lineage of microsporidia infecting fishes. We propose that these constitute a new genus, provisionally designated *Dictyocoela*, based on both structural and sequence information. Where sequence similarity was within 1%, isolates were considered to fall within the same species. Overall we designated six species, sequence divergence within this group is 4–11%, which is similar to the figure of 2–11% found within the genus *Nosema* (Baker *et al.* 1995). The remaining four sequences fall into discrete branches of the phylum Microspora. Two lie within the lineage infecting fishes, which primarily contains virulent parasites such as *Glugea* and *Spraguea* (Lom & Nilsen 2003). One species, *P. mulleri*, falls within the true *Pleistophora* clade (Terry *et al.* 2003) whereas the second, designated *Microsporidium* G, lies basal to the group containing *Spraguea lophii*. *Microsporidium* H, which had weak homology to existing sequence data, lies basal to this lineage, while *Microsporidium* I falls within a heterogeneous lineage containing the human infective species *Enterocytozoon* and *Endoreticulatus*.

In addition to the species identified from our systematic screen, VT has been noted to form as an element of the life cycle within many genera including *Encephalitozoon*, *Amblyospora*, *Edhazardia*, *Pleistophora* and *Flabelliforma* (Dunn & Smith 2001). These vertically transmitted microsporidia are not restricted to insects but have been reported from a wide range of hosts including bryozoans, molluscs, crustaceans, fishes and mammals. We have mapped

documented instances of VT and find that this trait has a widespread distribution throughout the phylum Microspora (figure 2). Vertically transmitted microsporidia occur in all lineages, suggesting either that this trait evolves with peculiar frequency or that it forms an ancestral element of the life cycle.

The relationship between VT and sex-ratio distortion is unlikely to be revealed without specific screening. Our finding, that five out of eight vertically transmitted microsporidian species tested in our amphipod screen have significant female bias in their distribution, suggests that sex-ratio distortion is taking place with high frequency. Sex-ratio distortion may result either from feminization of putative male offspring or from male killing. It is possible to discriminate between these two mechanisms via breeding experiments. We have recently demonstrated feminization by measuring the sex ratio of *G. duebeni* offspring from a population infected with *N. granulosis* and *D. duebenum*. Although infected and uninfected mothers produced equal numbers of offspring and survival was unaffected by the parasite, infected mothers produced significantly more daughters (Ironside *et al.* 2003*b*). The fact that both microsporidian species feminize crustacean hosts fits with observations on *Wolbachia*, which also induces feminization in crustacean hosts (Bouchon *et al.* 1998). These data imply that the mechanism of sex-ratio distortion is defined by the host sexual-differentiation pathway. In support of this we have recently demonstrated that *N. granulosis* blocks differentiation of the 'masculinizing' androgenic gland (Rodgers-Grey *et al.* 2004). This parallels observations on vertically transmitted *Wolbachia* infection in the isopod crustacean *Armadillidium vulgare* (Rigaud 1997), suggesting that these two phylogenetically distant parasites feminize their hosts by a common mechanism.

The ability of parasites to distort host sex ratio also occurs in diverse branches of the phylum. The feminizing parasites *N. granulosis* and *D. duebenum* are separated by one of the deepest divisions within the phylum. In addition to these feminizers, microsporidia such as *Amblyospora stimuli* and *Parathelohania anophelis* (Andreadis & Hall 1979) have been shown to cause sex-ratio distortion via male killing in dipteran hosts. These data suggest that sex-ratio distortion has arisen several times with at least two origins of feminization and a separate origin of male killing. The widespread distribution of vertically transmitted microsporidia across host phyla and the existence of sex-ratio distorters in multiple lineages argue for a wider evaluation of their interaction with their hosts.

Microsporidia are regarded as derived members of the fungi that are highly adapted to their parasitic lifestyle. Their reduced genome (Katinka *et al.* 2001) and relictual mitochondrion (Williams *et al.* 2002) suggest a peculiarly high level of dependence on the host, which might indicate selection towards mutualism. There are many parallels between these eukaryotic parasites and bacterial endosymbionts. Some characteristics, such as a genome reduction, may be attributed to their intracellular lifestyle, but others, including interaction with the host cytoskeleton, biased segregation during embryogenesis (Terry *et al.* 1999*b*), reduced pathogenesis and targeting of gonadal tissue (Terry *et al.* 1997), could be considered adaptations to VT. The widespread use of VT within the Microspora and the abilities of diverse species to modify host sex

(Andreadis & Hall 1979; Terry *et al.* 1999*b*; Ironside *et al.* 2003*b*) suggest that, like their bacterial counterparts, they are able to manipulate host sexual differentiation and, therefore, represent an important selective force in the evolution of host sex determination (Caubet *et al.* 2000; Charlat *et al.* 2003).

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