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Microsporidian parasites feminise hosts without paramyxean co-infection: support for convergent evolution of parasitic feminisation



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ABSTRACT

Feminisation of amphipod crustaceans is associated with the presence of at least three microsporidian parasites and one paramyxean parasite, suggesting that the ability to feminise has evolved multiple times in parasites of amphipods. Co-infection by a paramyxean with one of the putative microsporidian feminisers, *Dictyocoela duebenum*, has inspired the alternative hypothesis that all feminisation of amphipods is caused by paramyxia and that all microsporidian associations with feminisation are due to co-infection with paramyxia (Short et al., 2012). In a population of the amphipod *Gammarus duebeni*, breeding experiments demonstrate that the microsporidia *D. duebenum* and *Nosema granulosis* are associated with feminisation in the absence of paramyxia. Co-infection of the two microsporidia is no more frequent than expected at random and each parasite is associated with feminisation in the absence of the other. These findings support the original hypothesis that the ability to feminise amphipods has evolved in microsporidia on multiple occasions. Additionally, the occurrence of a non-feminising strain of *D. duebenum* in *Gammarus pulex* suggests that different strains vary in their feminising ability, even within microsporidian species. The presence or absence of feminising ability in a particular microsporidian strain should not therefore be generalised to the species as a whole.

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1. Introduction

Feminisation of genetic males by maternally inherited parasites is widespread among arthropods, including butterflies (Hiroki et al., 2002), woodlice (Bouchon et al., 1998) and amphipod crustaceans (Bulnheim, 1978; Ginsburger-Vogel and Desportes, 1979a). Feminising parasites can alter the sex ratios of host populations (Ginsburger-Vogel, 1975; Bouchon et al., 2008), increasing or decreasing population growth rates and hence resilience to perturbations. They can also cause dramatic changes in the genetic sex determination mechanisms of their hosts, including local extinction of sex chromosomes and transitions from male to female heterogamety (Rigaud and Juchault, 1993). Most known feminising parasites are bacteria of the genus *Wolbachia* (Valette et al., 2013). However, among amphipod crustaceans, feminisation is associated with eukaryotic microsporidian and paramyxean parasites (Bulnheim, 1978; Ginsburger-Vogel and Desportes, 1979a; Terry et al., 1998; Ironside et al., 2003). Previous investigations have indicated the existence of multiple species of feminising microsporidia in amphipods (Ironside et al., 2003; Mautner et al.,

2007). However, a recent study (Short et al., 2012) suggests that some or all instances of apparent feminisation by microsporidia in amphipods may be due to co-infection with feminising paramyxean parasites.

The production of thelygenous broods containing abnormally high numbers of female offspring is associated with parasitic infection in the amphipod species *Orchestia gammarellus* (Ginsburger-Vogel and Desportes, 1979a), *Gammarus duebeni* (Bulnheim, 1978; Terry et al., 1998; Ironside et al., 2003) and *Corophium curvispinum* (Mautner et al., 2007). In these species, the total number of offspring produced by infected females is not reduced, suggesting that the parasites distort the sex ratio by feminising rather than killing infected male embryos. In the case of *O. gammarellus*, the feminising parasite is a paramyxean, *Paramarteilia orchestiae* (Ginsburger-Vogel and Desportes, 1979b). In *G. duebeni*, feminisation is attributed to two microsporidian parasites, *Nosema granulosis* (Terry et al., 1999) and *Dictyocoela duebenum* (Terry et al., 2004). A further two feminising microsporidia, *Octosporea effeminans* and *Thelohania hereditaria*, have been described (Bulnheim and Vavra, 1968; Bulnheim, 1971) but these are now thought to be synonymous with *N. granulosis* and *D. duebenum* (Jahnke et al., 2013). In *C. curvispinum* a third, undescribed microsporidian appears to be responsible for sex ratio distortion (Mautner et al., 2007). These three microsporidia are distantly related and belong to clades

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consisting predominantly of non-feminisers (Terry et al., 2004), suggesting that the ability to feminise amphipod hosts has evolved on at least four separate occasions, once in paramyxea and three times in microsporidia. *Nosema granulosis* and *D. duebenum* exhibit low pathogenicity (Ironside et al., 2003; Kelly et al., 2003) and efficient vertical transmission (Terry et al., 1998; Ironside et al., 2003), which occurs through female hosts only. They therefore have the potential to obtain significant benefits from feminising male hosts. In addition to the three host species mentioned above, parasitic feminisation by microsporidia has been suggested in a number of other amphipod hosts, on the basis of higher prevalence in adult females than in males (Terry et al., 2004; Ryan and Kohler, 2010). These studies are not supported by experimental evidence from brood sex ratios and so the observed female bias in parasite prevalence might be produced by other causes, such as parasite tissue specificity for the female gonad, killing of infected male embryos or ecological differences between males and females affecting the likelihood of infection.

In certain populations of the amphipod *Echinogammarus marinus*, a large proportion of intersex individuals occur (Ford et al., 2006). These may be functionally male or female but show secondary sexual characteristics of both sexes. Intersex individuals of both functional sexes exhibit higher prevalence of infection with the microsporidian parasite *D. duebenum* than do normal individuals of either sex. The prevalence of infection in intersexes also appears to be correlated with the prevalence in normal females (Ford et al., 2007). These observations have been used to support the hypothesis that intersexuality in *E. marinus* results from incomplete parasitic feminisation of male hosts by *D. duebenum* (Ford et al., 2006, 2007). Recently, a paramyxean parasite was discovered in *E. marinus* (Short et al., 2012). This parasite, known only from its 16S ribosomal DNA sequence, also occurs at higher prevalence in intersex individuals than in normal individuals and, intriguingly, shows levels of co-infection with *D. duebenum* significantly higher than expected, given the prevalence of the two parasites (Short et al., 2012). Given that the paramyxean *P. orchestiae* is associated with feminisation and intersexuality in *O. gammarellus* (Ginsburger-Vogel and Desportes, 1979a), this discovery opened the possibility that intersexuality in *E. marinus* may be caused by the paramyxean, rather than by *D. duebenum*. In *Orchestia aestuarensis*, Ginsburger-Vogel (1991) observed a stronger association of male intersexuality with paramyxean infection than with microsporidian infection and showed that transplanted *O. aestuarensis* tissue co-infected with paramyxea and microsporidia induced intersexuality in male *O. gammarellus*, even though only the paramyxean cells survived in the new host. Short et al. (2012) used these findings as a basis to speculate that other presumed cases of feminisation by microsporidia, such as those in *G. duebeni* (Ironside et al., 2003) and *C. curvispinum* (Mautner et al., 2007), might be due to undetected co-infection with a paramyxean.

Co-infection with a single paramyxean species offers an attractive explanation for the surprising discovery that several different species of microsporidia are associated with feminisation in *G. duebeni* (Ironside et al., 2003). This co-infection hypothesis might be viewed as more parsimonious in that the trait for feminisation would need to evolve only once (in paramyxea) rather than at least four times convergently (thrice in microsporidia and once in paramyxea). This hypothesis might also explain the finding that morphologically and genetically similar strains of microsporidia are associated with feminisation in some amphipod populations but not in others (Bulnheim, 1978; Terry et al., 2004), (Ironside, R.E., 2003). The diversity and evolution of feminising microsporidia (Ph.D. thesis). University of Leeds, Leeds, UK). This has been interpreted as evidence that microsporidia exhibit heritable variation in their ability to feminise (Bulnheim, 1978) but might alternatively

be due to paramyxean co-infection with some microsporidian strains but not others.

However, there remains convincing evidence in support of feminisation by microsporidia, at least in the host *G. duebeni*. The sexual phenotypes of *G. duebeni* infected with different microsporidian species respond differently to hormonal and environmental manipulations such as injections of androgenic hormone and changes in temperature or salinity (Bulnheim, 1977; Rodgers-Gray et al., 2004; Jahnke et al., 2013). These results could only be explained by paramyxean co-infection if each microsporidian species formed an exclusive association with a different paramyxean strain. Furthermore, the co-infection hypothesis is based upon evidence of near-total co-infection of an undescribed paramyxean parasite and a microsporidian described as *D. duebenum* in *E. marinus*, and the association of these parasites with intersex phenotypes in natural populations (Ford et al., 2006, 2007; Short et al., 2012). No direct evidence from breeding or transfection experiments for complete or incomplete sex reversal in infected, genetically male *E. marinus* has yet been produced. It is also not entirely clear that the parasite described as *D. duebenum* by Short et al. (2012) in *E. marinus* belongs to the same strain, or even the same species, as the parasite associated with feminisation in *G. duebeni* (Ironside et al., 2003), since its *ssrDNA* sequence is only 98.6% similar. If the co-infection hypothesis is to be considered seriously as an alternative explanation for microsporidian-associated feminisation in amphipods then it must be shown to operate in a well-characterised system, such as *G. duebeni*, in which there is convincing experimental evidence that parasite-induced feminisation actually occurs.

Short et al. (2012) have demonstrated that a paramyxean parasite occurs in at least one species of Gammaridean amphipod, that this parasite is associated with intersexuality and that it shows a strong pattern of co-infection with a microsporidian. Given the strong evidence that a paramyxean *P. orchestiae* causes feminisation in *O. gammarellus* (Ginsburger-Vogel and Desportes, 1979a), it is therefore necessary to test directly the hypothesis that some or all feminisation associated with microsporidia in *G. duebeni* results from co-infection with a paramyxean parasite.

2. Materials and methods

2.1. Screening of wild-caught *G. duebeni* females for parasites

Gammarus duebeni samples were collected using a hand net from beneath rocks in shallow streams where they crossed the tideline on shores consisting of sand with scattered rocks. Sixty-two precopula pairs of *G. duebeni* were collected from Fintray Bay (55°46'05"N, 4°56'16"W) on the Isle of Cumbrae, Firth of Clyde, Scotland in October 2000. Females were separated from males and placed in a freezer at –80 °C. DNA was then extracted from their gonad tissue using two extractions with phenol/chloroform and one extraction with chloroform. The quality of the DNA was tested using a PCR for the host mitochondrial Cytochrome Oxidase 1 (Cox1) gene, with the primers HCO2198 (forward) (5' TAACTTCAGGGTGACCAAAAAATCA 3') and LCO1490 (reverse) (5' GGTCAACAAATCATAAAGATATTGG 3') (Folmer et al., 1994). Successful amplification indicated the presence of high quality DNA. Each DNA sample was then subjected to two PCR screens for feminising microsporidia. The first was a single-stage PCR using the primers 285NF (5' CGGATAACGGTATTACTTT 3') and 1164NR (5' CATAACGGACCTGTTTAAT 3'), which amplifies an 879 bp fragment of DNA from *N. granulosis* but not *D. duebenum*. The second was a two-stage PCR using the primers pairs 254SF (5' ATCAGTTAGTAAGTAGGGTAAAGGCCTA 3') and 981R (5' TGGTAAGCTGTC CCGCTTGAGTC 3'), followed by 280SF (5' TTAGACGAATACGGG

TACGGGGAAT 3') and 964R (5' CGCGTTGAGTCAAATTAGCCGCA CAC 3'), which amplifies a 684 bp fragment of DNA from *D. duebenum* but not *N. granulosis*. The specificity of these reactions for *N. granulosis* and *D. duebenum* was tested and confirmed using the PCR-RFLP method of Hogg et al. (2002). The Cox1 PCR and all three microsporidian PCRs were performed with 30 cycles, an annealing temperature of 50 °C and an extension time of 1 min.

To the authors' knowledge, no paramyxean parasites have been discovered previously in *G. duebeni* and so no DNA sequences are available for any such parasites. In order to screen for potential feminising parasites in *G. duebeni*, primers were designed to amplify DNA from the putative feminising paramyxia found in *E. marinus* and *Orchestia* spp. The rationale was that primers able to amplify the DNA of paramyxia from gammarid and talitrid hosts should be sufficiently general to detect related paramyxia in novel gammarid hosts. Primers for paramyxean parasites designed by Short et al. (2012) did not provide consistent results and so a range of new primers were designed on the basis of published paramyxean ssrDNA sequences available in GenBank ([JQ673484](#), [GU132549](#), [AJ250699](#)). These were tested for reliable amplification of paramyxean DNA from genomic DNA of an infected *E. marinus* individual (provided by Alex Ford, University of Portsmouth, UK). The most effective primer combinations were then used to screen samples from infected populations of *O. aestuarensis* and *O. gammarellus*, collected from beneath stones and *Spartina anglica* wrack in a salt marsh near Dale, Pembrokeshire, UK (51°43'22"N, 5°9'50"W).

For amplification of paramyxean DNA from *E. marinus* and *Orchestia* spp., the best results were provided by primers JIparaF3 (5' GATCAACGGGAGCGGT 3') and JIparaR3 (5' GCCCATCGGCAGAGGTAT 3') which amplify a 391 bp fragment of the paramyxean ssrDNA and by JIparaF1 (5' GGACCATGCTGAGACTAAA 3') and JIparaR1 (5' GAGTTCACGAGAAACAGTTG 3') which amplify a 980 bp fragment of the paramyxean ssrDNA. Both primer sets were used to screen the 62 adult female *G. duebeni* in PCRs with 45 cycles at an annealing temperature of 50 °C and with an extension time of 1 min.

The 95% confidence intervals for the prevalence of each parasite at Fintray Bay were calculated using the binomial distribution (Clopper and Pearson, 1934). A Chi-squared test, based on a 2 × 2 contingency table, was used to test for excesses or deficits in the number of individuals co-infected with multiple parasites with a *P* value of less than 0.05 considered to be significant.

2.2. Sex ratios of infected and uninfected *G. duebeni* and *G. pulex* females

An additional 34 precopula pairs of *G. duebeni* were collected from three sites on the Isle of Cumbrae on two occasions, October 1998 and May 2010. The sites were Fintray Bay, Stinking Bay (55°47'29"N, 4°54'45"W) and Sheriff's Port (55°45'29"N, 4°56'46"W). Each of these pairs was maintained in a separate container with 150 ml of brackish water to which rotted oak leaves and marine green algae were added for food and shelter. In order to eliminate sex ratio variability due to environmental sex determination, which is known to occur in *G. duebeni* (Dunn et al., 1993), a constant temperature of 12 °C and a constant photoperiod of 16 h light and 8 h dark were maintained throughout the experiment. At Sheriff's Port, the freshwater amphipod *Gammarus pulex* was discovered in the same stream as *G. duebeni*, with ranges slightly overlapping. Pairs of *G. pulex* were collected from this site and maintained under the same conditions as *G. duebeni* except that fresh water was used rather than brackish water.

After each pair had mated, the male was removed from the container and the female was maintained as before until her young were

released from her brood pouch. Each young amphipod was then moved to a separate container and allowed to grow for 5 months. At this point, each animal was anaesthetised using carbonated water and examined under a light microscope. Males were identified by the presence of penial papillae while females were identified by the presence of oostegites (brood plates). No intersex individuals (with both penial papillae and oostegites) were discovered. After sexing, young *G. duebeni* and *G. pulex* were frozen at –80 °C.

At least two offspring from each brood were dissected and DNA was extracted from gonad tissue using two extractions with phenol/chloroform and one extraction with chloroform (samples collected in 1998) or the Qiagen Blood and Tissue kit (Germany) according to the manufacturer's instructions (samples collected in 2010). The samples were subjected to PCR to screen for microsporidian and paramyxean infections as described in Section 2.1.

The 95% confidence intervals for the number of females produced in broods infected with each parasite species, as well as uninfected broods, were calculated using the binomial distribution (Clopper and Pearson, 1934). A Chi-squared test, based on a 2 × 3 contingency table, was used to test for differences between observed and expected numbers of females produced in *G. duebeni* broods infected with the *N. granulosis* and *D. duebenum* in comparison to uninfected broods. Separate Fisher's exact tests, based on 2 × 2 contingency tables, were used to test for differences between observed and expected numbers of females infected with each of the two microsporidian parasites, in comparison to uninfected broods of *G. duebeni* and *G. pulex*. In all cases, *P* values less than 0.05 were considered to be significant.

2.3. Phylogenetic analysis of *D. duebenum* sequences

A longer (1246 bp) fragment of *D. duebenum* ssrDNA was amplified from three of the infected *G. duebeni* and three of the infected *G. pulex* used in the breeding experiment, using the primers V1F and 1492R (Hogg et al., 2002) in PCRs with 45 cycles at an annealing temperature of 50 °C and with an extension time of 1 min. PCR products were sequenced using an ABI3730xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA). These sequences were aligned using Clustal W (Thompson et al., 1994) with all other *Dictyocoela* sequences on the National Center for Biotechnology Information (NCBI), USA nucleotide database which covered 90% or more of their length. These included the *D. duebenum* reference sequence, other published *D. duebenum* sequences from *G. duebeni* and *E. marinus*, and outgroup sequences from *Dictyocoela muelleri*, *Dictyocoela diporeiae*, *Dictyocoela berillonum*, *Dictyocoela gammarellum*, *Dictyocoela deshayesum* and *Dictyocoela cavimanum* (Table 1).

A phylogenetic tree was constructed using Bayesian inference in MrBayes (Huelsenbeck and Ronquist, 2001). A maximum likelihood test of 24 different nucleotide substitution models, implemented in Mega 6 (Tamura et al., 2013) indicated that the general time reversible model, with gamma-distributed rate variation and a proportion of invariant sites (GTR + I + G) provided the best fit to the data according to the Akaike Information Criterion (corrected) and so this model was used. A tree search was conducted over 120,000 generations, sampling every 10 generations, with a burn in of 3000 generations.

3. Results

3.1. Screening of wild-caught *G. duebeni* females for parasites

Of the 62 adult female *G. duebeni* from the Fintray Bay population that were screened by PCR, 16 were infected with *N. granulosis* only, six were infected with *D. duebenum* only and six were

Table 1
Dictyocoela ssrDNA sequences used to construct the Bayesian phylogenetic tree (Fig. 3).

GenBank Accession number	Parasite species	Host species	Location
AF397404	<i>Dictyocoela duebenum</i>	<i>Gammarus duebeni</i>	Isle of Cumbrae, Scotland
FN434091	<i>Dictyocoela duebenum</i>	<i>Gammarus duebeni</i>	Iceland
JQ673482	<i>Dictyocoela duebenum</i>	<i>Echinogammarus marinus</i>	Portsmouth, England
HM991451	<i>Dictyocoela</i> sp.	<i>Gammarus pseudolimnaeus</i>	Kalamazoo County, USA
JQ673483	<i>Dictyocoela duebenum</i>	<i>Echinogammarus marinus</i>	Inverkeithing, Scotland
KP091740	<i>Dictyocoela</i> sp.	<i>Gammarus pulex</i>	Isle of Cumbrae, Scotland
AJ438956	<i>Dictyocoela muelleri</i>	<i>Gammarus roeseli</i>	Dijon, France
FN434090	<i>Dictyocoela muelleri</i>	<i>Gammarus duebeni</i>	River Aulne, France
AJ438955	<i>Dictyocoela muelleri</i>	<i>Gammarus duebeni</i>	Loch Neagh, Northern Ireland
GU196256	<i>Dictyocoela</i> sp.	<i>Gammarus lacustris</i>	Loch Kinardochy, Scotland
GU196258	<i>Dictyocoela</i> sp.	<i>Gammarus fossarum</i>	Soběšice, Czech Republic
KF537632	<i>Dictyocoela diporeiae</i>	<i>Diporeia</i> sp.	Lake Superior, USA
JQ673481	<i>Dictyocoela berillonum</i>	<i>Echinogammarus marinus</i>	Portsmouth, England
AJ438957	<i>Dictyocoela berillonum</i>	<i>Echinogammarus berilloni</i>	Poitiers, France
KF830272	<i>Dictyocoela berillonum</i>	<i>Dikerogammarus haemobaphes</i>	Poland
AJ438960	<i>Dictyocoela cavimanum</i>	<i>Orchestia cavimana</i>	Isle of Cumbrae, Scotland
AJ438959	<i>Dictyocoela cavimanum</i>	<i>Talitrus saltator</i>	Ile d'Oleron, France
AJ438961	<i>Dictyocoela deshavesum</i>	<i>Talorchestia deshavesum</i>	Ile d'Oleron, France
AJ438958	<i>Dictyocoela gammarellum</i>	<i>Orchestia gammarellum</i>	Isle of Cumbrae, Scotland

infected with *N. granulosis* and *D. duebenum*. A chi-squared contingency test indicated that the number of *G. duebeni* co-infected with both microsporidian parasites did not differ significantly from the number expected (4.26) given the prevalence of the two parasites ($\chi^2 = 1.37$, degrees of freedom (d.f.) = 1, $P > 0.05$). No infections with paramyxean parasites were detected and a 95% confidence interval of 0–0.06 was calculated for paramyxean prevalence (Fig. 1).

3.2. Sex ratios of infected and uninfected *G. duebeni* and *G. pulex* females

Of the 34 broods of offspring produced by *G. duebeni* pairs in the laboratory, four were infected with *D. duebenum* and 12 were infected with *N. granulosis*. All 16 infected broods were extremely female biased while all uninfected broods were male biased, to varying degrees (Table 2, Fig. 2). A Chi-squared test indicated that the number of female offspring produced in broods infected with microsporidia differed significantly from the number expected if infected and uninfected broods did not differ with regard to sex ratio ($\chi^2 = 153$, d.f. = 2, $P < 0.001$). Fisher's exact tests indicated that significant female biases in broods infected with *N. granulosis* ($P < 0.0001$) and *D. duebenum* ($P < 0.0001$), in comparison to uninfected broods. Paramyxean infection was not detected in any of the *G. duebeni* broods.

Mortality of *G. pulex* in the laboratory was higher than that of *G. duebeni* and breeding was less successful. Of the seven females

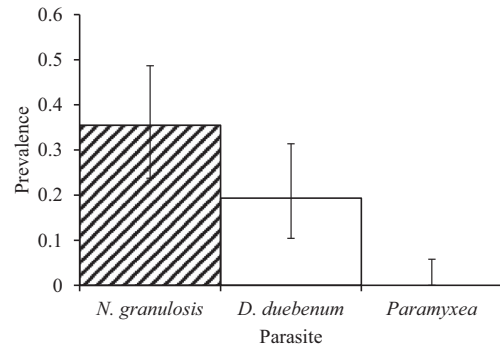


Fig. 1. Prevalence of the putative feminising parasites *Nosema granulosis*, *Dictyocoela duebenum* and paramyxia in a population of *Gammarus duebeni* on the Isle of Cumbrae, Scotland. Error bars indicate 95% binomial confidence intervals for parasite prevalence.

Table 2
 Male and female offspring produced under laboratory conditions by *Gammarus duebeni* and *Gammarus pulex* females. All broods were tested by PCR for infection with *Nosema granulosis*, *Dictyocoela duebenum* and paramyxia.

	Male	Female
<i>G. duebeni</i>		
<i>N. granulosis</i> (12 broods)	3	89
<i>D. duebenum</i> (4 broods)	0	24
Paramyxia	0	0
Uninfected (18 broods)	125	36
<i>G. pulex</i>		
<i>N. granulosis</i>	0	0
<i>D. duebenum</i> (5 broods)	16	4
Paramyxia	0	0
Uninfected (2 broods)	4	1

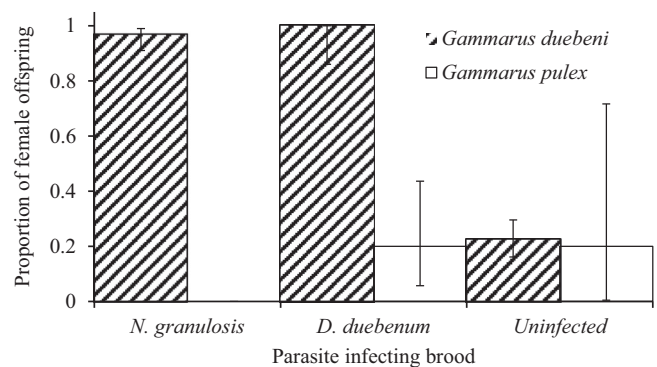


Fig. 2. Sex ratios produced by *Gammarus duebeni* and *Gammarus pulex* females, uninfected and infected with the microsporidian parasites *Nosema granulosis* and *Dictyocoela duebenum*.

which produced broods of offspring, five were infected with *Dictyocoela* sp. Both infected and uninfected females produced male-biased broods of offspring. A Fisher's exact test indicated no significant difference between the sex ratios produced by infected and uninfected females ($P = 1$).

3.3. Phylogenetic analysis of *D. duebenum* sequences

All *Dictyocoela* ssrDNA sequences from Isle of Cumbrae *G. duebeni* matched the *D. duebenum* reference sequence (Genbank: AF397404) exactly. This sequence remains the only *Dictyocoela* sequence associated unambiguously with feminisation. All *Dictyocoela* ssrDNA sequences from Isle of Cumbrae *G. pulex* were

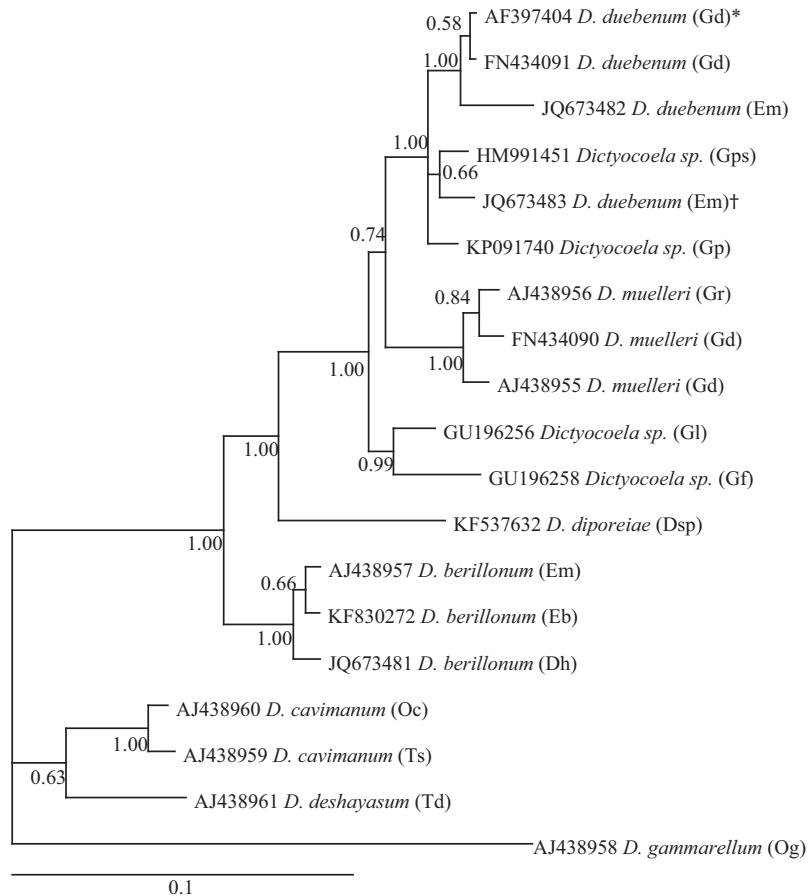


Fig. 3. Bayesian phylogenetic tree of *Dictyocoela* ssrDNA sequences. Gd, *Gammarus duebeni*; Gf, *Gammarus fossarum*; Gl, *Gammarus lacustris*; Gp, *Gammarus pulex*; Gps, *Gammarus pseudolimnaeus*; Gr, *Gammarus roeseli*; Eb, *Echinogammarus berilloni*; Em, *Echinogammarus marinus*; Dsp, *Diporeia* sp.; Dh, *Dikerogammarus haemobaphes*; Oc, *Orchestia cavimana*; Og, *Orchestia gammarellus*; Ts, *Talitrus saltator*; Td, *Talorchestia deshayesi*. **Dictyocoela duebenum* reference sequence. †*Dictyocoela duebenum* sequence associated with intersexuality in *E. marinus* (Short et al., 2012).

identical (GenBank: **KP091740**) but differed from the *D. duebenum* reference sequence by 16 substitutions (1.29%) and one gap. The ssrDNA sequence of *Dictyocoela* amplified from *E. marinus* from Inverkeithing, Scotland (Short et al., 2012) and associated with intersexuality differs from the reference *D. duebenum* sequence by 16 single nucleotide substitutions (1.37%) and two gaps.

All ssrDNA sequences described as *D. duebenum* and included in the Bayesian phylogenetic analysis are found within a single, well-supported clade (Fig. 3), which also includes a sequence described as *Dictyocoela* sp. from the North American amphipod *Gammarus pseudolimnaeus* (Ryan and Kohler, 2010). Within this clade, the reference *D. duebenum* sequence falls within a well-supported subclade containing sequences from *G. duebeni* (from Scotland, Isle of Man and Iceland) and a single sequence from *E. marinus*, from a sample collected near Portsmouth, England (Yang et al., 2011). The *D. duebenum* sequence associated with intersexuality of *E. marinus* at Inverkeithing (Short et al., 2012) falls outside of this subclade, as do the sequences from *G. pulex* and *G. pseudolimnaeus*.

4. Discussion

A PCR-based screen of adult female *G. duebeni* on the Isle of Cumbrae, Scotland did not detect any evidence of infection with paramyxean parasites. In contrast, the microsporidian parasites *N. granulosis* and *D. duebenum* were both present at prevalences of 0.19 and 0.35, respectively. Breeding experiments indicated that *G. duebeni* females infected with *N. granulosis* or *D. duebenum* produce extremely female-biased broods in the absence of

co-infecting paramyxean parasites, while uninfected females produce male-biased broods. Hence, this study does not support the hypothesis suggested by Short et al. (2012), that apparent feminisation by microsporidia is caused by co-infecting paramyxean parasites.

The PCR-based screening method employed in this study was designed to detect paramyxean parasites similar to those associated with intersexuality and feminisation in *E. marinus* and *Orchestia* spp., respectively. It could therefore be argued that feminisation in *G. duebeni* might be caused by the co-infection of microsporidia with a more divergent, and therefore undetected, paramyxean parasite. However, given that numerous histological studies have also revealed no evidence of paramyxean parasites in *G. duebeni*, there seems little reason, at present, to doubt that feminisation in *G. duebeni* is caused by microsporidia.

Furthermore, although the microsporidia *N. granulosis* and *D. duebenum* are observed to co-infect the same hosts, the incidence of co-infection is not significantly higher than expected given the prevalence of the two parasites. It is also clear, from this study and others (Ironside et al., 2003), that each microsporidian parasite is able to cause feminisation in the absence of the other. Given that *N. granulosis* and *D. duebenum* are phylogenetically distant and that each has close, non-feminising relatives, this study supports the hypothesis that the ability to feminise crustacean hosts has evolved at least twice among the microsporidia and at least once among the paramyxia.

Due to the apparent absence of paramyxean parasites from *G. duebeni* on the Isle of Cumbrae, this study could not test the

hypothesis that paramyxean parasites cause feminisation in gammarid amphipods, as suggested by Short et al. (2012). It also remains uncertain whether *D. duebenum* causes feminisation in *E. marinus*. Both hypotheses could be tested by performing breeding experiments on *E. marinus*, similar to those conducted on *G. duebeni* in this study.

All cases in which *D. duebenum* is unambiguously associated with feminisation through breeding experiments, in this and previous studies (Ironside et al., 2003), involve parasites sharing a single *ssrDNA* sequence, the *D. duebenum* reference sequence (GenBank: AF397404). Parasites with this sequence are widespread among British and Scandinavian populations of *G. duebeni* (Wilkinson et al., 2011). The *D. duebenum* strain associated with intersexuality in *E. marinus* differs from the feminising reference strain by 1.37%. This *E. marinus* strain appears most closely related to a strain obtained from the North American host species *G. pseudolimnaeus* (Ryan and Kohler, 2010). The *G. pseudolimnaeus* strain has higher prevalence in females than in males, suggesting that it may be a feminiser, but this has not been confirmed through breeding experiments. Ryan and Kohler (2010) do not note any association between this parasite and intersexuality in *G. pseudolimnaeus*.

On the Isle of Cumbrae, *G. pulex* is infected with a *D. duebenum* strain with an *ssrDNA* sequence which differs from the reference sequence by 1.29%. Breeding experiments provide no indications of feminisation by this *D. duebenum* strain in *G. pulex*. All infected *G. pulex* broods contained at least one male and the overall sex ratio of infected individuals was male-biased and similar to the sex ratio of uninfected offspring. In contrast, the offspring produced by *G. duebeni* from the Isle of Cumbrae that were infected with the reference *D. duebenum* strain were entirely female. These findings indicate that even closely related strains of *D. duebenum* may differ in their ability to feminise infected hosts and suggest that it should not be assumed that all strains of *D. duebenum* are feminisers. Conversely, if one strain of *D. duebenum* is shown not to feminise its host, this does not necessarily cast doubt on the feminising ability of other strains, especially when they occur in different host species. Theoretical models also indicate that the evolution of feminisation is likely to be highly dynamic, depending upon the relative effectiveness of horizontal and vertical transmission (Ironside et al., 2011), and so may vary between parasite populations and host species.

In conclusion, evidence provided in this study and elsewhere supports the hypothesis that the microsporidia *N. granulosis* and *D. duebenum* cause feminisation of *G. duebeni*. There is currently no evidence that feminisation associated with microsporidia in *G. duebeni* is caused by co-infecting paramyxean parasites. The phenomenon observed by Short et al. (2012) of higher than expected levels of co-infection of *D. duebenum* and a paramyxean parasite in *E. marinus* is surprising and warrants further investigation, regardless of whether one, both or neither parasite is capable of feminising its host. However, the hypothesis that the ability to feminise crustacean hosts has arisen several times in microsporidian and paramyxean parasites by convergent evolution provides the best explanation for the data currently available.

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