

# Associations between mycophagous *Drosophila* and their *Howardula* nematode parasites: a worldwide phylogenetic shuffle

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## Abstract

Little is known about what determines patterns of host association of horizontally transmitted parasites over evolutionary timescales. We examine the evolution of associations between mushroom-feeding *Drosophila* flies (Diptera: Drosophilidae), particularly in the *quinaria* and *testacea* species groups, and their horizontally transmitted *Howardula* nematode parasites (Tylenchida: Allantonematidae). *Howardula* species were identified by molecular characterization of nematodes collected from wild-caught flies. In addition, DNA sequence data is used to infer the phylogenetic relationships of both host *Drosophila* (mtDNA: COI, II, III) and their *Howardula* parasites (rDNA: 18S, ITS1; mtDNA: COI). Host and parasite phylogenies are not congruent, with patterns of host association resulting from frequent and sometimes rapid host colonizations. *Drosophila*-parasitic *Howardula* are not monophyletic, and host switches have occurred between *Drosophila* and distantly related mycophagous sphaerocerid flies. There is evidence for some phylogenetic association between parasites and hosts, with some nematode clades associated with certain host lineages. Overall, these host associations are highly dynamic, and appear to be driven by a combination of repeated opportunities for host colonization due to shared breeding sites and large potential host ranges of the nematodes.

*Keywords:* coevolution, cospeciation, *Drosophila*, mycophagy, parasitism, phylogeny

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## Introduction

The extent to which associated organisms coevolve depends, among other things, on the duration of the association. A major recent goal of comparative biology has been to determine whether phylogenies of associated organisms, such as hosts and parasites or symbionts (Brooks 1988; Huelsenbeck *et al.* 1997, 2000), and plants and pollinators or herbivores (Farrell & Mitter 1990; Weiblen 2001), are generally congruent with each other. Congruence indicates that such associations transcend speciation events and are therefore relatively old (e.g. Whitfield 2002). Many factors can lead to incongruence of host and parasite phylogenies, including colonization of new host species

(host switching events), parasite extinction or release of certain hosts or host populations from parasitism (sorting events), and parasite speciation in the absence of host speciation (duplication events) (Page 1994).

The most important determinant of host and parasite congruence is parasite transmission mode (Herre *et al.* 1999). Most cases of phylogenetic congruence between hosts and symbionts are those where the symbiont is vertically transmitted (i.e. from mother to offspring) within the host population (but see Nishiguchi *et al.* 1998). For example, microbial endosymbionts of aphids and deep-sea clams exhibit congruent phylogenies with their hosts (Moran *et al.* 1995; Peek *et al.* 1998). Congruent phylogenies can also arise when the parasite has very little opportunity for dispersal to new hosts, as has been found in pocket gopher chewing lice (Hafner & Nadler 1988). However, even these highly restrictive kinds of associations can

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depart from perfect congruence, as has been shown in the vertically transmitted *Wolbachia*, which are bacterial reproductive parasites of arthropods (Werren *et al.* 1995), and seabird chewing lice (Paterson *et al.* 2000).

The majority of parasites however, are horizontally transmitted (i.e. not strictly from mother to offspring), and there are several reasons why their phylogenies are unlikely to be congruent with those of their hosts. Many parasites infect more than one host species (Woolhouse *et al.* 2001). Taxonomically related parasites can infect distantly related hosts (Siddiqi 2000), which can occur only via host switching events. Novel host-parasite associations in the wild, such as emerging diseases, are also increasingly common (Daszak *et al.* 2000). Nevertheless, all parasites, including horizontally transmitted ones, must successfully locate, infect, develop and reproduce within their hosts. This could require such a degree of host-specific adaptation that one would expect parasites to be restricted to certain host lineages, with few switches to distantly related hosts. A comparison of host and parasite phylogenies can be used to estimate the frequency of host switches in horizontally transmitted parasites, as well as the degree to which such shifts are constrained by host phylogeny (Ricklefs & Fallon 2002). In conjunction with experimental infections (Nishiguchi *et al.* 1998), this approach may also help us to understand what determines the origin and fate of novel infections.

In this study, we examine the evolution of associations between *Howardula* (Tylenchida: Allantonematidae) parasitic nematodes and their host *Drosophila* (Diptera: Drosophilidae) flies. These parasites are transmitted horizontally between hosts, with much opportunity for colonization of novel host species over evolutionary time scales. We first assess the diversity of these *Howardula* by molecular characterization of nematodes obtained from wild-caught flies. We then determine and compare the phylogeny of *Drosophila*-parasitic *Howardula* with that of their hosts, and ask the following questions: (i) Are host and parasite phylogenies congruent, indicating long-term lineage-specific associations? (ii) Are *Drosophila*-parasitic *Howardula* monophyletic, or can interspecific colonization transcend boundaries of dipteran families?

Obligate parasitism of insects has evolved multiple times in nematodes (Blaxter *et al.* 1998), including at least once in the largely plant-parasitic order Tylenchida. Arthropod-parasitic tylenchids infect insects from at least six orders, including beetles, flies, wasps and bees, fleas, thrips, and true bugs, as well as mites (Siddiqi 2000). Two patterns appear to characterize host associations in this group, although their taxonomy and systematics are largely understudied: host switches to taxonomically and ecologically diverse hosts, and subsequent specialization and close association with certain host clades. Nematodes of the genus *Howardula* infect diverse beetles and flies, with the greatest number of described species infecting chrysomelid

leaf beetles (Elsey 1977; Poinar *et al.* 1998). There are currently 18 valid described species of *Howardula* (Zakharenkova 1996; Poinar *et al.* 1998), although this must be a great underestimate, as parasites are discovered and their host associations determined only by dissecting adult insects.

Mushroom-feeding Diptera also represent a large source of *Howardula* diversity, with nematodes reported from the families Drosophilidae (Gillis & Hardy 1997), Phoridae (Richardson *et al.* 1977), Sphaeroceridae, and Sepsidae (J. Jaenike, unpublished data). *Drosophila* flies, particularly from the closely related *quinaria* and *testacea* species groups, are some of the most abundant insect visitors to decaying fleshy mushrooms in temperate and boreal forests (Grimaldi & Jaenike 1984; Kimura & Toda 1989; Wertheim *et al.* 2000). These *Drosophila* are commonly infected by *Howardula*, and their associations are well characterized in North America, Europe and Japan (Kimura & Toda 1989; Jaenike 1992; Gillis & Hardy 1997). There are currently only two described species of *Drosophila*-parasitic *Howardula* (Welch 1959; Poinar *et al.* 1998), but, as we show below, this is an underestimate (see also Jaenike 1996).

*Drosophila*-parasitic *Howardula* are direct parasites (i.e. no intermediate hosts), and can often have severe effects on host fitness, including complete sterility of females of some species (Jaenike 1992). Inseminated female nematodes infect fly larvae by piercing through their cuticle (Welch 1959). When the adult fly emerges, the nematode mother-worm begins releasing juveniles into the haemocoel of the host. These are passed from the anus and ovipositor of the host as it visits mushrooms, where the nematodes subsequently mate and continue the cycle. Females that are not sterilized by *Howardula* disperse both nematodes and offspring into mushrooms, and therefore a small fraction of parasite transmission is potentially vertical (Jaenike 2000). However, because individual mushrooms are often oviposited on by multiple adult *Drosophila*, belonging to several species (Jaenike & James 1991), there are generally ample opportunities for horizontal transmission of parasites both within and among host species. We therefore predict specialization of parasites with certain lineages of hosts, as expected by constraints of parasite adaptation, but not parallel cladogenesis of *Drosophila* and *Howardula*.

## Materials and methods

### *Taxon sampling and DNA extraction*

*Howardula nematodes.* Our study includes all *Howardula* (both described and undescribed species) that have been reported to infect *Drosophila*, except for two undescribed species, one that infects the cactophilic *repleta* group species *D. nigrospiracula* in the Sonoran desert (Polak 1993), and one that infects the *quadrivittata* species group and *D. histrio* in Japan (Kimura & Toda 1989) (Table 1). We also

consider three *Howardula* species that parasitize insects other than *Drosophila*, including two species obtained from mycophagous *Leptocera* sp. (Diptera: Sphaeroceridae) and *H. dominicki*, a parasite of the tobacco flea beetle, *Epitrix hirtipennis* (Coleoptera: Chrysomelidae). Nematodes were collected by dissecting wild-caught adults, collected from North America, Europe, and Japan. Single motherworms were frozen upon dissection from hosts, and DNA was subsequently extracted using the DNeasy™. Tissue Kit from Qiagen Inc. (protocol for animal tissues).

*Drosophila*. We include all known *Drosophila* hosts of *Howardula* (Table 2), except for the cactophilic species *D. nigrospiracula* (Polak 1993), and three Palearctic species in the *quadrivittata* species group (*Hirtodrosophila* radiation), *D. sexvittata*, *D. trivittata* and *D. trilineata* (Kimura & Toda 1989). All host *Drosophila* in our study breed primarily on mushrooms, except *D. pseudoobscura*. The breeding habits of *D. pseudoobscura* are not well known, but it has been reported to breed in sap fluxes (Carson 1951) and acorns (Spieth 1987). We also include nine *quinaria* group species that are not known to harbour *Howardula*, including five nonmycophagous species, for a total of 20 (out of 28 described) *quinaria* group species. All four members of the *testacea* group are included.

#### Sequencing and phylogenetic analysis

*General*. Nematode DNA sequencing was carried out on an ABS 377 sequencer at the Genomic Analysis and Technology

Core (GATC) at the University of Arizona, USA. Fly DNA sequencing was carried out at San Francisco State University, USA, on a Catalyst 800 Molecular Biology Laboratory Station. For all species, we sequenced DNA in both directions, and checked for contamination by performing BLAST searches (Altschul *et al.* 1997) (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences were first aligned in CLUSTALW (Thompson *et al.* 1994) (<http://www2.ebi.ac.uk/clustalw>) using default settings, and then manually aligned in MACCLADE 4.0.1. (Maddison & Maddison 2001). We used PAUP\*4.0b6 (Swofford 2001) for all phylogenetic analyses.

*Nematode ITS1*. For initial molecular typing of nematodes, we sequenced the internal transcribed spacer (ITS1) region of rDNA, which has been advocated as a useful marker for closely related nematode species (Powers *et al.* 1997). We used the rDNA2 and rDNA1.58s primers described in Powers *et al.* (1997) and the following PCR conditions: 2.5 mM dNTPs (0.4 µL/reaction), 10 µM primers (0.6 µL), 50 mM MgCl<sub>2</sub> (0.6–1 µL), 10X buffer (2 µL), Taq polymerase (0.1 µL), genomic DNA (1 µL), water (14.3–14.7 µL). Amplification proceeded for 35 cycles, with 1 min each of denaturation (94 °C), annealing (56 or 57 °C) and extension (72 °C). Due to rapid sequence evolution, we were only able to align ITS1 sequence of a subset of nematodes. We constructed a neighbour-joining (NJ) tree for these taxa, but did no further phylogenetic analysis for this region.

*Nematode mtDNA*. In order to better resolve one clade of closely related *Drosophila* parasites (the '*Drosophila*-parasite

**Table 1** *Howardula* nematodes included in phylogenetic analysis

Nematode designation	Host species from which sample was obtained	Sample number	Collection locale	rDNA accession	mtDNA accession
<i>H. aoronymphium</i>	<i>Drosophila phalerata</i>	E329, E334, E336 E2F	Denmark The Netherlands	AF519224	AF519209
	<i>D. falleni</i>	N66, N124, N399	New York	AF519229	AF519210
	<i>D. neotestacea</i>	N90	Virginia		AF519211
		N101	Pennsylvania		AF519212
<i>H. cf. aoronymphium</i>	<i>D. orientacea</i>	J308, J316, J344	Japan	AF519225	AF519213
<i>H. neocosmis</i>	<i>D. acutilabella</i>	AC314, AC340	Florida	AF519226	AF519218
<i>H. cf. neocosmis</i>	<i>D. munda</i>	MU397, MU398	Arizona	AF519227	
		SU321, SU322	Washington	AF519228	AF519217
<i>Howardula</i> sp. F	<i>D. falleni</i>	F125, F165, F167	New York	AF519222	AF519214
<i>Howardula</i> sp. B	<i>D. brachynephros</i>	B301, B331, B332	Japan	AF519223	AF519215, 216
<i>Howardula</i> sp. MA	<i>D. macroptera</i>	MA381, MA382	Arizona	AF519233	
<i>Howardula</i> sp. PS	<i>D. pseudoobscura</i>	PS99	California	AF519231	AF519220
<i>Howardula</i> sp. SPA	<i>Leptocera</i> sp. (Sphaeroceridae)	SP392	Washington	AF519232	
		SP2, SP361, SP363	New York		AF519219
<i>Howardula</i> sp. SPB	<i>Leptocera</i> sp. (Sphaeroceridae)	SP391	Washington	AF519230	
<i>H. dominicki</i>	<i>Epitrix hirtipennis</i> (Coleoptera)	C348, C350	N. Carolina	AF519234	AF519221

**Table 2** *Drosophila* species included in the study and their *Howardula* parasites. For collection locales, strain numbers refer to strains from *Drosophila* species stock centre (<http://stockcentre.arl.arizona.edu/>). GenBank accession numbers AF147105-147134 are sequences obtained from Spicer & Jaenike (1996)

Species group	Species (and native nematode)	Collection locale	COI accession	COII accession	COIII accession
<i>testacea</i>	<i>D. neotestacea</i> ( <i>H. aoronymphium</i> †)	New York	AF519395	AF519331	AF519363
	<i>D. orientacea</i> ( <i>H. cf. aoronymphium</i> ‡)	Japan	AF519398	AF519334	AF519366
	<i>D. putrida</i> ( <i>H. aoronymphium</i> †)	New York	AF519399	AF519335	AF519367
	<i>D. testacea</i> ( <i>H. aoronymphium</i> *)	Germany	AF519405	AF519341	AF519373
<i>quinaria</i>	<i>D. brachynephros</i> ( <i>Howardula</i> sp.¶)	Japan	AF519382	AF519318	AF519350
	<i>D. curvispina</i> ( <i>Howardula</i> sp.¶)	Japan	AF519384	AF519320	AF519352
	<i>D. deflecta</i>	New Jersey	AF319385	AF519321	AF519353
	<i>D. falleni</i> ( <i>H. aoronymphium</i> † <i>Howardula</i> sp.‡)	New York, (15130–1961.0)	AF147106–7	AF147116–7	AF147126–7
	<i>D. guttifera</i>	Texas, Florida (15130–1971.0, 1971.1)	AF147108–9	AF147118–9	AF147128–9
	<i>D. innubila</i>	Arizona	AF519389	AF519325	AF519357
	<i>D. kuntzei</i> ( <i>H. aoronymphium</i> *)	The Netherlands	AF519390	AF519326	AF519358
	<i>D. limbata</i>	The Netherlands	AF519391–2	AF519327–8	AF519359–60
	<i>D. munda</i> ( <i>H. cf. neocosmis</i> §)	Arizona	AF519394	AF519330	AF519362
	<i>D. nigromaculata</i>	Japan	AF519396	AF519332	AF519364
	<i>D. occidentalis</i>	California	AF519397	AF519333	AF519365
	<i>D. palustris</i>	New York	AF147112	AF147122	AF147132
	<i>D. phalerata</i> ( <i>H. aoronymphium</i> *)	The Netherlands	AF147105	AF147115	AF147125
	<i>D. quinaria</i>	New York	AF147114	AF147124	AF147134
	<i>D. recens</i> ( <i>H. aoronymphium</i> †)	New York	AF147113	AF147123	AF147133
	<i>D. suboccidentalis</i> ( <i>H. cf. neocosmis</i> **)	Oregon, California	AF519400–3	AF519336–9	AF519368–71
	<i>D. subpalustris</i>	South Carolina (15130–2071.0.1)	AF147110–1	AF147120–1	AF147130–1
	<i>D. subquinaria</i>	Washington	AF519404	AF519340	AF519372
	<i>D. transversa</i> ( <i>H. aoronymphium</i> *)	The Netherlands	AF519406	AF519342	AF519374
	<i>D. unispina</i> ( <i>Howardula</i> sp.¶)	Japan	AF519408	AF519344	AF519376
<i>cardini</i>	<i>D. acutilabella</i> ( <i>H. neocosmis</i> **)	Florida	AF519381	AF519317	AF519349
	<i>D. cardini</i>	Florida	AF519383	AF519319	AF519351
<i>tripunctata</i>	<i>D. tripunctata</i>	Tennessee	AF519407	AF519343	AF519375
<i>immigrans</i>	<i>D. immigrans</i> ( <i>H. aoronymphium</i> *)	New York	AF519388	AF519324	AF519356
<i>histrion</i>	<i>D. histrion</i> ( <i>Howardula</i> sp.¶)	Japan	AF519386	AF519322	AF519354
<i>macroptera</i>	<i>D. macroptera</i> ( <i>Howardula</i> sp.§)	Arizona	AF519393	AF519329	AF519361
<i>quadriovittata</i>	<i>D. histrioides</i> ( <i>Howardula</i> sp.¶)	Japan	AF519387	AF519323	AF519355
<i>busckii</i>	<i>D. busckii</i>	Costa Rica (13000–0081.0)	AF519411	AF519347	AF519379
<i>obscura</i>	<i>D. affinis</i>	Nebraska (14012–0141.0)	AF519410	AF519346	AF519378
	<i>D. pseudoobscura</i> ( <i>Howardula</i> sp.§)	Arizona (14011.0212.0)	AF519412	AF519348	AF519380
	<i>D. subobscura</i>	Washington	AF519409	AF519345	AF519377

\*Gillis & Hardy (1997); †Jaenike (1992); ‡Jaenike (1996); §Jaenike and Perlman (unpublished); ¶Kimura & Toda (1989);

\*\*Poinar *et al.* (1998).

ITS1' clade), and because we did not have a suitable outgroup for the rapidly evolving ITS1 sequence, we sequenced a portion of mitochondrial cytochrome c oxidase subunit I, using primers developed by Folmer *et al.* (1994) and described in Sukhdeo *et al.* (1997). We used the PCR protocol described above, but with an annealing temperature of 50 °C. We were unable to amplify mtDNA from *Howardula* infecting *D. macroptera* (MA) and from one *Howardula* species infecting *Leptocera* (SPB). Phylogenetic analyses were carried out as above, except that there were no gaps. Because mtDNA evolves rapidly in nematodes and is only recommended for phylogenetic reconstruction of closely related taxa (Blouin *et al.* 1998), we tested for DNA saturation at first, second and third position transitions and transversions, and we used the *Howardula* from one *Leptocera* parasite (SPA) as the outgroup. We compared genetic distances (estimated from our ML model) with the number of changes between pairs of taxa, with nonlinear relationships suggesting saturation.

*Nematode 18S.* We sequenced the 18S small subunit of ribosomal DNA, using the primers described in Blaxter *et al.* (1998) (<http://nema.cap.ed.ac.uk/biodiversity/sourhope/nemoprimer.html>). PCR conditions were as above. As outgroups, we used the tylenchid nematodes *Subanguina radicola* (GenBank Accession No. AF202164) and *Pratylenchoides magnicauda* (AF202157), which were sequenced by Felix *et al.* (2000) and had high similarity to *Howardula* in BLAST searches. Under maximum parsimony (MP), we performed heuristic searches with TBR branch swapping and 1000 random addition replicates. Maxtrees were set to increase without limit. Gaps were treated as a new state, and there were no gaps larger than 4 bases. All characters were weighted equally. We assessed clade robustness by bootstrap analysis (Felsenstein 1985), using heuristic searches with 5000 replicates and a random addition sequence of  $n = 1$ . We also estimated the 18S phylogeny using maximum likelihood analysis. We used a general time reversible model of nucleotide substitution, with rate heterogeneity between sites (GTR +  $\Gamma$  + I). We used NJ and MP trees to estimate the six nucleotide transition parameters, the gamma shape parameter for rate heterogeneity ( $\Gamma$ ), and the proportion of invariable sites (I). We performed a heuristic search with TBR branch swapping, a stepwise addition starting tree, and the asis stepwise addition option.

We used SH tests (Shimodaira & Hasegawa 1999; Goldman *et al.* 2000) to ask whether tree topologies in which *Howardula* that parasitize *Drosophila* were constrained to be monophyletic were significantly different (i.e. less likely) than the ML topology. We compared the ML tree with the 30 highest scoring constraint trees obtained in a search using the parameters estimated for the ML tree. SH tests were implemented in PAUP\*. The test compares the difference in log-likelihoods between the best (ML)

and alternate trees with a distribution of test statistics generated from 1000 nonparametric bootstrap replicates, using the resampling estimated log-likelihood (RELL) technique (Goldman *et al.* 2000).

*Drosophila mtDNA.* *Drosophila* DNA extraction methods are described in Spicer (1995). We obtained DNA sequences from the mitochondrial cytochrome oxidase I, II, and III subunits (COI-III). The corresponding mtDNA sequences from *D. yakuba* and *D. melanogaster*, which were used as outgroups in this study, came from Clary & Wolstenholme (1985) and de Bruijn (1983), respectively. We also used *D. affinis*, *D. subobscura* and *D. busckii* as additional outgroups. The amplification primers for COI, C1-N-2191 and C1-J-1751, were made specific to *Drosophila*, and can be found in Spicer (1995). Most of the COII primers appeared in Liu & Beckenbach (1992), although some (TK-N-3785 and TL2-J-3037) have been modified from the original compilation (Spicer 1995). The amplification primers for COIII, C3-J-5014 and C3-N-5460, appeared in Simon *et al.* (1994). Most of the internal sequencing primers were designed independently and can be found in Spicer (1995). PCR conditions are as in Spicer (1995). Phylogenetic analyses were carried out as above (see nematode 18S), except that any gaps were coded as missing data. The few gaps in this dataset were due to regions at the ends of subunits for which we were not able to obtain the sequence.

*Determination of parasite host range.* The presence of cryptic *Howardula* species complicates determinations of host range. Host associations for all *Howardula* in this study were determined by molecular characterization of isolates obtained from wild-caught flies, except for the generalist *H. aoronymphium*. We obtained sequence from *H. aoronymphium* collected from three of its nine known host species: *D. falleni*, *D. neotestacea* and *D. phalerata*. In experimental laboratory infections (Perlman & Jaenike 2003), we confirmed that *H. aoronymphium* can successfully infect all other reported hosts: *D. immigrans*, *D. kuntzei*, *D. putrida*, *D. recens*, *D. testacea*, and *D. transversa*. It was previously shown that in North America, the generalist *H. aoronymphium* consists of a single epidemiological unit and is not comprised of host races (Jaenike & Dombeck 1998).

*Congruence of host and parasite phylogenies.* We visualized host and parasite phylogenies and used the method of reconciled trees in TREEMAP 1.0b (Page 1995) to test for incongruence. This method aligns host and parasite cladograms such that the number of cospeciation events (matching nodes) is maximized. For perfect congruence, the number of cospeciation events is the number of internal nodes minus one (Page 1994). We then created 1000 random associations by reshuffling host and parasite taxa, calculating the maximum number of cospeciation events

for each reshuffled association and comparing their distribution. Random trees were generated in TREEMAP using the proportional-to-distinguishable model option. TREEMAP requires fully resolved trees; we therefore performed analyses for all possible resolutions of clades with low bootstrap values. We included only *Drosophila* and their parasites in the analysis.

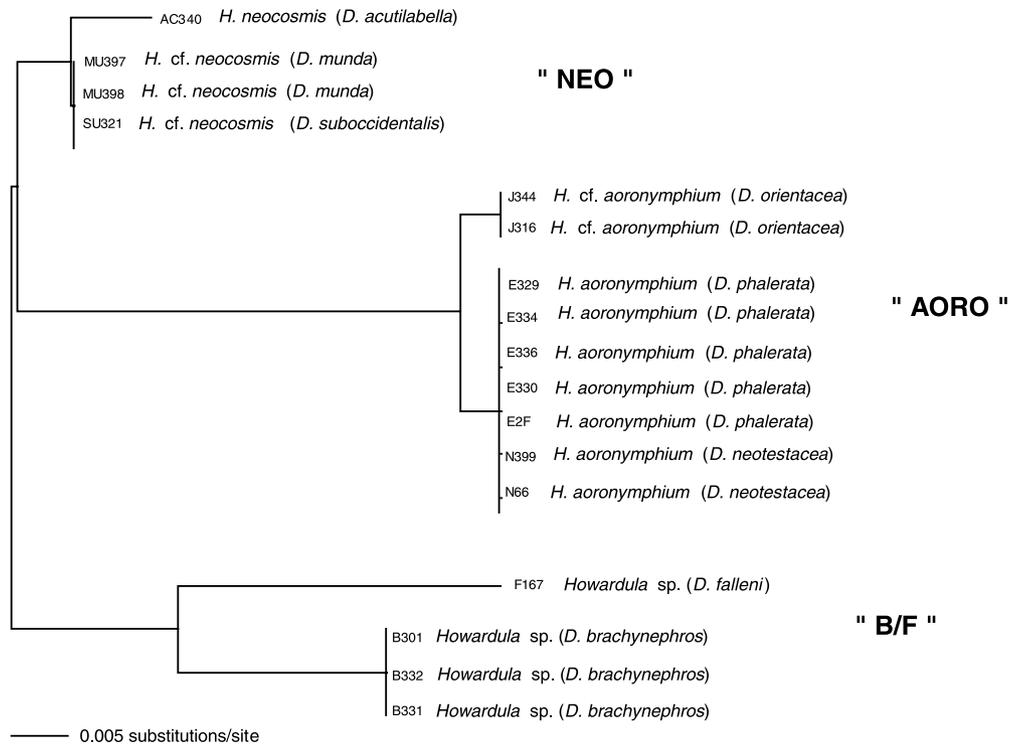
## Results

### Howardula phylogeny

*Nematode ITS1*. ITS1 sequence revealed a total of eight distinct lineages of *Drosophila*-parasitic *Howardula*, and two distinct *Leptocera*-parasitic species. ITS1 was highly AT-biased (80%). Sequence length for all nematodes was 230–310 base pairs, except for one sphaerocerid parasite (SPA: 501 bp) and *H. dominicki* (C: 400 bp). We were only able to align ITS1 for a subset of nematodes. This group consisted of all *Drosophila*-parasitic *Howardula*, except for the two species infecting *D. macroptera* (MA) and *D. pseudoobscura* (PS). A neighbour-joining tree of the '*Drosophila*-parasite ITS1' clade reveals three distinct groupings (Fig. 1): (i) *H. aoronymphium* from Europe and America, which are identical, and a closely related Japanese nematode (the

AORO group); (ii) *H. neocosmis* from North America and a very close sister species (the NEO group); and (iii) two undescribed species, one from North America and one from Japan (the B/F group). This NJ tree was constructed after removal of one 45–70 bp AT-rich region of ambiguous alignment. We were also able to align the *D. pseudoobscura* (PS) parasite sequence with that of one sphaerocerid parasite (SPB) after removing a 50–66 and an 8–18 bp region, but we could not align this pair with any other sequences.

*Nematode mtDNA*. Our complete aligned data set consisted of 382 bp. MtDNA was AT-rich (66%), and only third position transitions appear saturated (data not shown). This data set does not resolve the relationships between the three main groupings of the '*Drosophila*-parasite ITS1' clade (AORO, NEO, B/F). ML analysis produced a tree of score  $-\ln 1283.65$ , with the following parameters of nucleotide substitution: A-C = 2.15, A-G = 11.24, A-T = 1.32, C-G = 0.44, C-T = 7.23, G-T = 1, I = 0.44,  $\Gamma = 0.78$ , and with NEO and AORO as sister groups. This tree score was not significantly different from the MP topologies ( $-\ln 1283.78$ – $1285.05$ ,  $P > 0.3$ ). MP analysis yielded three MPRs (treelength = 180, CI = 0.83, 71 parsimony informative characters), which differed in the placement of *Howardula* sp. B and *Howardula* sp. F, and which were only one step shorter than the ML



**Fig. 1** Neighbour-joining tree, using ITS1 region sequences for the *Howardula* clade termed '*Drosophila*-parasite ITS1', with midpoint rooting. This clade comprises three species complexes, denoted AORO, B/F and NEO. Sequences are 282–292 bp long. We were unable to align the other *Howardula* ITS1 sequences.

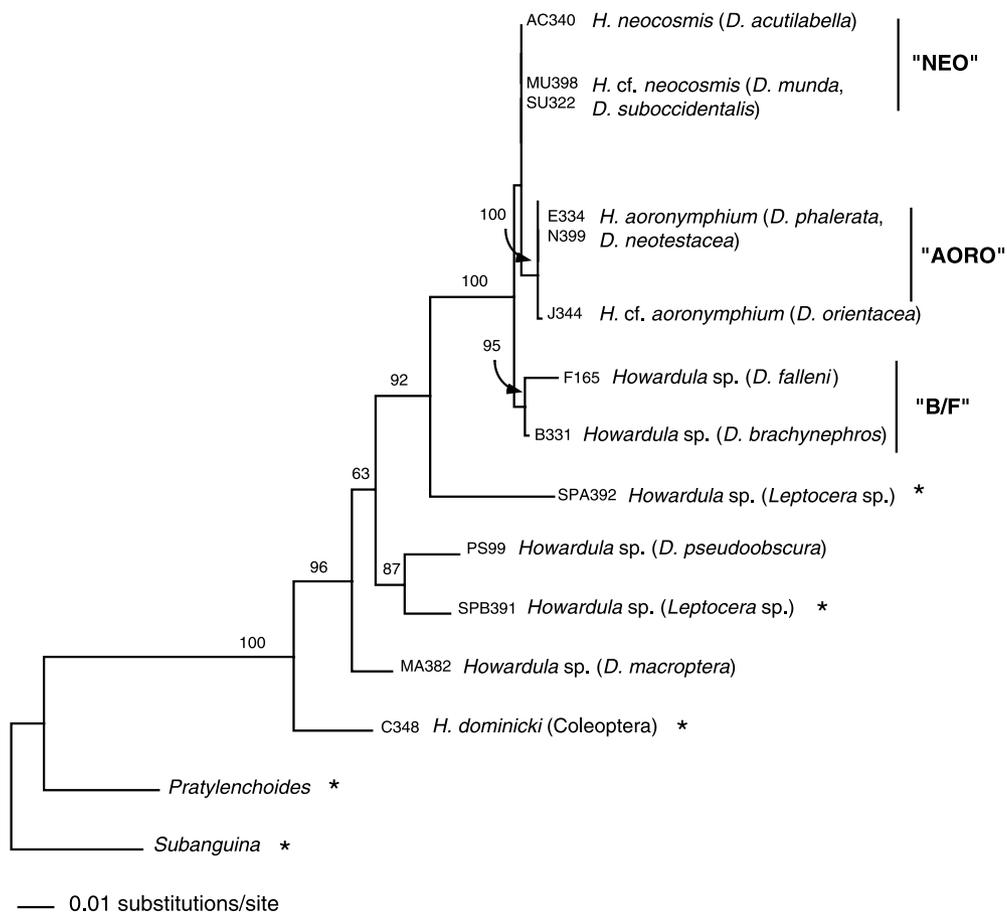
and NJ topologies. The NJ topology is the same as the one produced from the ITS1 analysis.

**Nematode 18S.** Sequence lengths per nematode species ranged from 1733 to 1791 bp. We were unable to sequence 530 bases at the 5' end for one of the sphaerocerid parasites (SPB). Only 1630 bases of 18S sequence were available for our two outgroups. Our complete aligned data set was therefore 1125 characters long. MP, NJ and ML analyses produced similar tree topologies (Fig. 2). MP analysis produced 4 most parsimonious trees (treelength = 348, CI = 0.80, 140 parsimony informative characters). These differed in their resolution of the 'Drosophila-parasite ITS1' clade. ML analysis produced a tree of score  $-\ln 3112.5$ , which was identical in topology to one of the most parsimonious trees. The following parameters of nucleotide substitution were used in the ML analysis: A-C = 0.9, A-G = 3.96, A-T = 2.28, C-G = 0.21, C-T = 7.43, G-T = 1, I = 0.55,  $\Gamma = 0.9$ . The ML tree was significantly different from the 30 best trees having the constraint

that *Drosophila* parasites are monophyletic ( $P < 0.02$ ). The best constrained tree had a score of  $-\ln 3130.69$ , for a difference in likelihood scores of 18.19 (SH test:  $P = 0.016$ ). Thus, the *Drosophila*-parasitic species of *Howardula* are not monophyletic.

**Nematode species delineation.** We use a combination of pairwise sequence divergence (using ML distances), experimental infections, and morphological differences to delineate nematode species. At 18S, *Howardula* isolates/species MA, PS, SPA, and SPB exhibit 2–5% sequence divergence from their nearest neighbours; these distances are greater than, or within the range of, interspecific 18S divergence found within the nematode genera *Caenorhabditis* (0.8–1.8%; Fitch *et al.* 1995), *Heterorhabditis* (0–1%; Liu *et al.* 1997) and *Steinernema* (1–7%, Liu *et al.* 1997).

Within the closely related 'Drosophila-parasitic ITS1' clade, mtDNA sequence divergence between the three species groups 'AORO', 'B/F', and 'NEO', ranges from 16 to 25%. These species groups also show morphological and



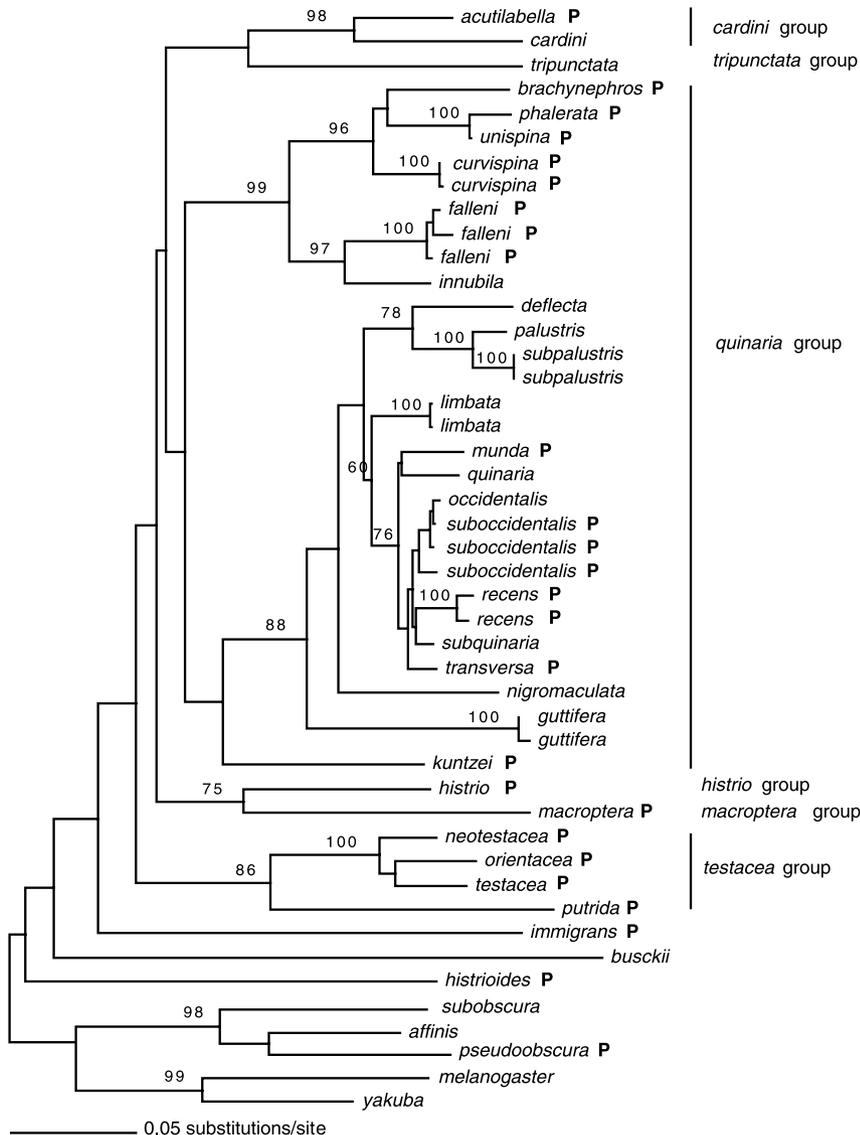
**Fig. 2** Maximum likelihood tree, using *Howardula* 18S sequences, rooted with the nematodes *Pratylenchoides magnicauda* and *Subanguina radiccicola*. Aligned data set is 1125 characters. Topology is identical to one of 4 most parsimonious trees. Numbers above branches indicate parsimony percent bootstrap values. Asterisks indicate nematodes that do not infect *Drosophila*.

potential host range differences (Perlman & Jaenike 2003). *Howardula* sp. B and F, and *H. aoronymphium* and *H. cf. aoronymphium* exhibit 20% and 10% mtDNA sequence divergence, respectively. In a survey of nematode mtDNA divergence, Blouin *et al.* (1998) found that interspecific divergences range from 10 to 20%, and intraspecific divergences from 0 to 7%. In experimental laboratory infections, *H. aoronymphium* and *H. cf. aoronymphium* also show significantly different potential host ranges (Perlman & Jaenike 2003). Finally, *H. neocosmis* and *H. cf. neocosmis* exhibit 5% mtDNA divergence, and show quantitative morphological differences (Poinar *et al.* 1998). In laboratory infections, *H. neocosmis* infected its native host *D. acutilabella* at significantly higher rates than *D. munda*, the host of *H. cf. neocosmis* (S. Perlman, unpublished data). In summary, these analyses indicate the presence of eight

*Drosophila*-parasitic species of *Howardula* among the isolates we have examined: *H. aoronymphium*, *H. cf. aoronymphium*, *H. neocosmis*, *H. cf. neocosmis*, and *Howardula* species 'B', 'F', 'MA', and 'PS'.

*Drosophila* mtDNA phylogeny. The sequenced regions of the mitochondrial cytochrome oxidase gene encompass a 413 base pair (bp) segment of subunit I (*D. yakuba* positions 1778–2190; total length of subunit is 1535 bp), the entire subunit II (*D. yakuba* positions 3083–3766) comprising 688 bp, and a 416-bp segment of subunit III (*D. yakuba* positions 5015–5430; total length of subunit is 788). The *D. yakuba* positions refer to the Clary & Wolstenholme (1985) sequence.

ML analysis of the COI, II and III dataset produced a tree of score  $-\ln 12619.5$  (Fig. 3), with the following parameters



**Fig. 3** Maximum likelihood tree using *Drosophila* COI, II and III sequences. Numbers above branches indicate parsimony percent bootstrap values. (None of the nodes where MP trees differ from the ML tree have strong bootstrap support.) Sequences are 1517 bp long. Species that are known to be infected by *Howardula* in the wild are labelled P. Note that some uninfected species have not been sampled intensively enough to exclude the possibility of parasitism.

of nucleotide substitution: A-C = 3.48, A-G = 41.98, A-T = 24.33, C-G = 8.02, C-T = 110.25, G-T = 1, I = 0.59,  $\Gamma$  = 1.12. MP analysis produced 12 most parsimonious trees (treelength = 2421, CI = 0.33, 467 out of 1518 parsimony informative characters) (trees not shown). These trees differ in the following places: (i) in the grouping of the closely related *quinaria* group species *D. munda*, *D. quinaria*, *D. recens*, *D. transversa*, *D. suboccidentalis* and *D. subquinaria*; (ii) in the ordering of *D. nigromaculata* and *D. guttifera*; and (iii) in the relationship between the *testacea*, *quinaria* and (*histrio* + *macroptera*) species groups. None of the nodes where the MP trees differ from the ML tree have strong bootstrap support. In all analyses, *D. suboccidentalis* appears paraphyletic with respect to *D. occidentalis*; these are almost indistinguishable morphologically and might not be reproductively isolated. With respect to species group relationships, only the (*cardini* group + *tripunctata* group) and the (*histrio* group + *macroptera* group) pairings are consistently supported. Branches joining these lineages with the *quinaria* and *testacea* groups are short, and no combinations are significantly different than the species group topology obtained in the ML tree (SH tests:  $P > 0.05$ ).

Nematode parasitism is distributed throughout the *quinaria* and *testacea* groups. Most species (9 out of 10) for which there are no records of *Howardula* parasitism belong to one clade (the '*recens-guttifera*' clade) of the *quinaria* group (Fig. 3). However, most of the uninfected species have not been sampled adequately to rule out the presence of parasites.

*Congruence of Drosophila and Howardula phylogenies.* Host and parasite phylogenies are not congruent (Fig. 4). The maximum number of matching nodes (cospeciation events) inferred from the reconciliation analysis was 3 (perfect congruence would be 8). All resolutions of clades with low bootstrap support yielded the same number of cospeciation events. The inferred number of cospeciation events was not significantly different from that obtained by randomizing parasite taxa across *Drosophila* species, which yielded  $1.68 \pm 0.03$  SE. ( $P = 0.14$ ) cospeciation events. However, this number was significantly different from one obtained by randomizing host taxa, which yielded  $0.51 \pm 0.02$  ( $P = 0.006$ ) cospeciation events. The reconstruction with the fewest steps yielded 3 cospeciation events, 5 host switches, 0 duplication events and 25 sorting events.

## Discussion

Our study reveals little congruence between *Howardula* and *Drosophila* phylogenies, indicating frequent colonizations of new host species. Such colonization requires at least two conditions. First, there must be opportunities

for transmission of nematodes between species. Most of the *Drosophila* in our study are generalists, utilizing many of the same species of mushrooms as breeding sites (Lacy 1984; Kimura & Toda 1989; Wertheim *et al.* 2000). More importantly, interspecific aggregation can be pronounced, and as many as four different *Drosophila* species can emerge from a single mushroom (Grimaldi & Jaenike 1984; Jaenike & James 1991). As mushrooms are the site of host infection by nematodes, these parasites will often encounter multiple potential host species.

The second requirement for a host shift is that the parasite be capable of infecting, developing, and reproducing in the new host. Thus, the new host must be similar, in certain critical respects, to the ancestral host. The present findings of widespread parasite colonization of phylogenetically distinct *Drosophila* species indicates that these flies are, to some extent, similar environments with respect to parasite adaptation. Such conditions (opportunities for interspecific transmission and host similarity) can favour host generalism by the parasites. *H. aoronymphium* is a good example; it successfully infects at least four species in North America (Jaenike 1992), and five in Europe (Gillis & Hardy 1997).

The degree of host switching that has occurred between distantly related families (specifically, sphaerocerids and drosophilids) was unexpected. As a consequence of such host shifts, *Drosophila*-parasitic *Howardula* are paraphyletic. The parasite of *D. pseudoobscura* (PS) is most closely related to one of the sphaerocerid parasites (SPB). The '*Drosophila*-parasitic ITS1' clade is distantly related to the two other *Drosophila*-parasitic species (MA and PS). Virtually nothing is known about the biology of these latter two *Howardula* species. Even the two sphaerocerid parasite species (SPA and SPB) are distantly related, even though both species can occur in the same individual fly. In addition to Drosophilidae and Sphaeroceridae, *Howardula* have been found in mushroom-feeding Phoridae (Richardson *et al.* 1977) and Sepsidae (J. Jaenike, unpublished data). The occurrence of *Howardula* in several families of mushroom-feeding flies, in conjunction with our phylogenetic data, highlights the ecological potential for host-switching between diverse hosts. It is important to emphasize that the *Howardula* in our study are not fly generalists. In laboratory experiments, we were unable to infect sphaerocerids with *Drosophila* parasites or *Drosophila* with sphaerocerid parasites (Jaenike 1992; S. Perlman, unpublished data).

The potential for rapid host switching and incorporation of novel hosts by *Howardula* is further suggested by the striking lack of genetic variation among American and European *H. aoronymphium*, even at the rapidly evolving ITS1 sequence. ITS1 and mitochondrial sequences of North American and European samples were found to be identical, suggesting that one (or both) of these populations are evolutionarily very young. It is likely that *H. aoronymphium*

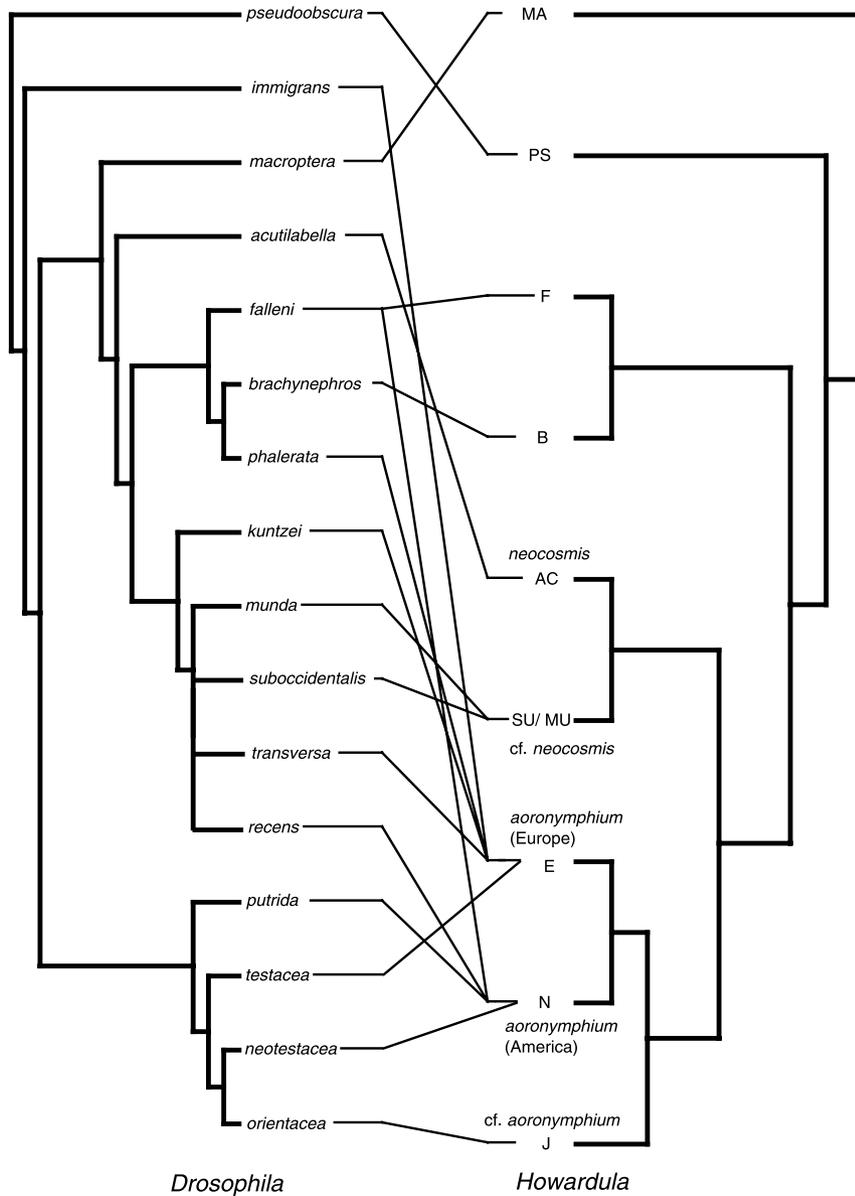


Fig. 4 Associations of *Howardula* nematodes and *Drosophila* species, as determined by host records in natural populations.

recently invaded North America and/or Europe and, in the process, must have incorporated an entirely new set of *Drosophila* into its host range. *H. neocosmis* and its very closely related sister species, although very similar at the molecular level, parasitize hosts belonging to different species groups in eastern and western North America. As with *H. aoronymphium*, this suggests a recent host shift.

Despite the great potential for horizontal transmission and ecological conditions favouring generalism, our study uncovered a number of *Howardula* that are host specialists. A few of these are cryptic species that were previously mistaken for a more common host-generalist nematode. For example, *Howardula* sp. F, the parasite of *D. falleni*, is unable to infect *D. neotestacea*, *D. putrida* or *D. recens* (Jaenike 1996; Perlman & Jaenike 2003); these four species share

mushrooms and all are parasitized by the generalist nematode *H. aoronymphium*. Indeed, *Howardula* sp. F was initially thought to be a host-restricted variant of *H. aoronymphium* (Jaenike 1996). In addition, at least two *Howardula* species from Japan, the 'orientacea specialist' (*H. cf. aoronymphium* J) and the nematode infecting *D. brachynephros* (*Howardula* sp. B) were previously identified as *H. aoronymphium* (Kimura & Toda 1989). In laboratory infections, the 'orientacea specialist' was unable to infect Japanese *quinaria* group species (Perlman & Jaenike 2002). In total, our molecular analysis identified 6 new *Drosophila*-parasitic *Howardula* species, and 2 new sphaerocerid-parasitic *Howardula* species.

There is some association between phylogenies of parasites and hosts in our analysis. This is demonstrated by the

fact that the maximum number of inferred cospeciation events is significantly more than that obtained by randomizing host taxa across parasite species (but not by randomizing parasite taxa across host species). This pattern could result from actual cospeciation of host and parasite lineages or from phylogenetic limitations on host shifts, so that parasites could only colonize closely related species of hosts (Ricklefs & Fallon 2002). This indicates that certain parasite clades tend to be associated with particular host lineages. For example, *H. aoronymphium*, which occurs in both Europe and North America, and its Japanese sister species are associated with all four species of the *testacea* group. In addition, the parasites of *D. brachynephros* (B) and *D. falleni* (F) are sister species, and their hosts are close relatives. We predict that the nematodes that infect *D. curvispina* and *D. unispina* (Kimura & Toda 1989) are the same species as infects *D. brachynephros*, since these hosts are all closely related members of the *D. quinaria* group.

What determines nematode host range in the wild? While *Howardula* parasitism is distributed throughout the *testacea* and *quinaria* groups, nine of the 10 species for which there are no records of parasitism occur in the 'recens-guttifera' clade of the *quinaria* group (Fig. 3). *Howardula* can infect and grow in seven of these 10 species in the laboratory (the other hosts were not tested), demonstrating that these species are intrinsically suitable as hosts for *Howardula* (Perlman & Jaenike 2003). It is possible that these species are infected at low rates in the wild, but that limited sampling has not been sufficient to find parasitized flies.

Alternatively, the lack of infection of species may be related to their breeding sites. For example, five of the 10 uninfected species, *D. deflecta*, *D. quinaria*, *D. limbata*, *D. subpalustris* and *D. palustris*, breed on decaying vegetation instead of mushrooms. It is not known why decaying vegetation breeders might not be infected in the wild. In a field experiment, it was shown that *H. aoronymphium* from laboratory-reared flies could be transmitted to *D. quinaria* breeding in decaying vegetation (J. Jaenike, unpublished data). It is possible that decaying vegetation breeders are not infected because they occur at densities too low to support a parasite population (Jaenike & Perlman 2002). Because breeding in decaying vegetation is clearly a derived condition among these flies (Spicer & Jaenike 1996), the loss of parasitism associated with such a breeding site shift would represent a sorting event, thus bringing about incongruence of host and parasite phylogenies.

In conclusion, our data reveal high levels of host switching and rapid incorporation of novel hosts across North America, Europe and Asia. These patterns are probably driven by the great potential for colonization of new hosts, due to shared host breeding sites, in combination with large potential host ranges (Perlman & Jaenike 2003), even for parasites that currently utilize only a few host species.

Thus, *Drosophila*–*Howardula* associations are highly dynamic over evolutionary time scales. We suspect that evolutionarily dynamic host ranges and lack of phylogenetic congruence are common, if not the rule, in associations characterized by horizontal transmission of parasites and ecological mingling of different host species, i.e. most host-parasite associations.

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- Steve Perlman's research focuses on the evolution and ecology of interspecific interactions, particularly between insects and diverse associated organisms, such as parasitic nematodes and bacterial symbionts. Greg Spicer's research interests focus on molecular approaches to diverse questions, including systematics of *Drosophila* and mites, and host-parasite coevolution. The main emphasis of DeWayne Shoemaker's research is in population and evolutionary genetics, focusing on fire ants and *Wolbachia*-insect interactions. John Jaenike's research focuses on internal challenges faced by mushroom feeding species of *Drosophila*, including interactions with nematode parasites, male-killing and CI *Wolbachia*, and X chromosome meiotic drive.
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