

# A SURVEY OF FUNGI WHICH KILL MICROSCOPIC ANIMALS IN THE DUNG OF THE AMAMI RABBIT

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Dung of the early-evolved Amami rabbit of Japan was surveyed for the presence of fungi which attack microscopic organisms. Several species of predacious nematophagous fungi and species endoparasitic in nematodes and rotifers were found. In addition, zygomycetous species attacking amoebae and tardigrades, and one flagellate zoospore species holocarpic in various host organisms, were found.

**Keywords:** nematode, rotifer, amoeba, tardigrade, nematophagous, endoparasitic, flagellate, zoospore, holocarpic.

The Amami Islands of Amami Ohshima and Tokunoshima which form part of the Ryukyu Archipelago are situated 127 - 129° E and 25 - 27° N in southern Japan; together the islands are less than 1000 km<sup>2</sup>.

The Amami rabbit (*Pentalagus furnessi* Stone), which is found only on the Amami Islands, is an indigenous species which is believed to have evolved in the Pliocene period (Dawson, 1981). It differs from more recently evolved lagomorphs in having small eyes and ears, short legs and long claws (Fig 1). The total population of the Amami rabbit on Amami Ohshima and Tokunoshima is estimated at around 4000 (Yamada, Sugimura & Koyanagi, 1995). The rabbits inhabit the forests and eat leaves of the Japanese pampas grass, *Miscanthus sinensis*, acorns of the pasania tree, *Castanopsis seibodia*, bark and bamboo shoots (Sugimura, 1990). They are nocturnal as their black colouration indicates. The Amami rabbit was designated a symbol of national heritage by the government in 1921 and this status was raised in 1963. Despite banning hunting of the rabbit, its numbers are declining due to deforestation and it is classified as endangered by the IUCN and USDI.

The remote geographical location and small size of the Amami Islands combined with the

early evolution and history of the Amami rabbit made an ecological survey of predacious fungi in the rabbit dung an interesting area for research.

Whilst many fungi subsisting on microscopic animals appear to be ubiquitous, others have, as yet, a very restricted reported geographical distribution and many are known to exhibit a high degree of host-specificity. Predacious and parasitic fungi attack microscopic animals such as nematodes, rotifers, tardigrades and amoebae. Such fungal species are found in all the major mycological groups and they display a wide variety of trapping and infection methods. Many are host-specific and host-dependent whilst others are able to exploit several host organisms and some have a saprophytic capability. The nematode and rotifer species which are attacked are mainly bacteriophagous and are found in habitats where decomposition of organic matter is taking place. In such habitats rich in bacteria, the nematode and rotifer populations increase. At such times, fungi which subsist on these animals, either host-dependently or as a supplement, are able to grow and propagate. Dung is usually a good source of such fungal species as it retains moisture well, is full of bacteria and harbours a diversity of microscopic animals.

## Methods

A total of 16 collections of dung of the Amami rabbit were made from Amami Ohshima on three occasions in autumn, winter and summer. Collections were taken from Sumiyo Village, Yamato Village, Kawauchi and Mt Naon. Dung samples were mixed with distilled water and several drops of the liquid were placed on to Petri plates containing corn meal agar (CMA). More distilled water was added to the plates after a few days to assist breeding of nematodes and rotifers. Predacious hyphomycetous fungi were routinely isolated and obtained in pure culture using Raper's rings. Endoparasitic species were isolated and cultured using methods previously

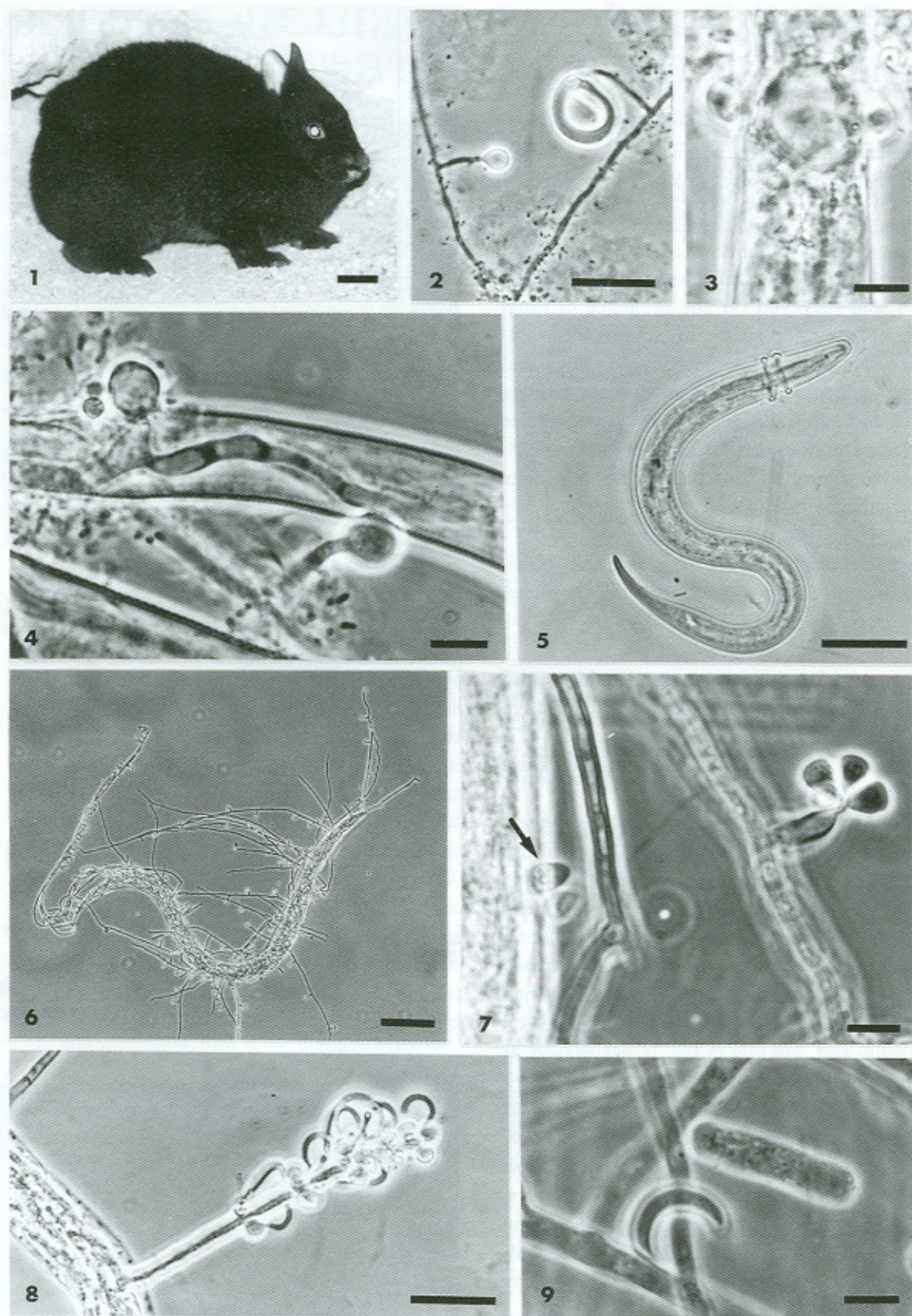


Fig 1 Amami rabbit (Scale bar, 40 mm). Fig 2 Passive ring and stalked knob of *Dactylaria candida* (Scale bar, 20  $\mu$ m). Fig 3 Infection bulb inside the nematode body (Scale bar, 6  $\mu$ m). Fig 4 Knobs of *D. candida* producing assimilative hyphae in the nematode body (Scale bar, 6  $\mu$ m). Fig 5 Nematode with passive rings of *D. candida* (Scale bar, 40  $\mu$ m). Fig 6 *Plectus* nematode infected with *Verticillium balanoides* (Scale bar, 50  $\mu$ m). Fig 7 Conidia of *V. balanoides*. Conidium sticking to nematode cuticle (arrow) (Scale bar, 6  $\mu$ m). Fig 8 Sporulation of *Harposporium cycloides* from nematode (Scale bar, 20  $\mu$ m). Fig 9 Infection conidium and arthroconidium in *H. cycloides* (Scale bar, 6  $\mu$ m).

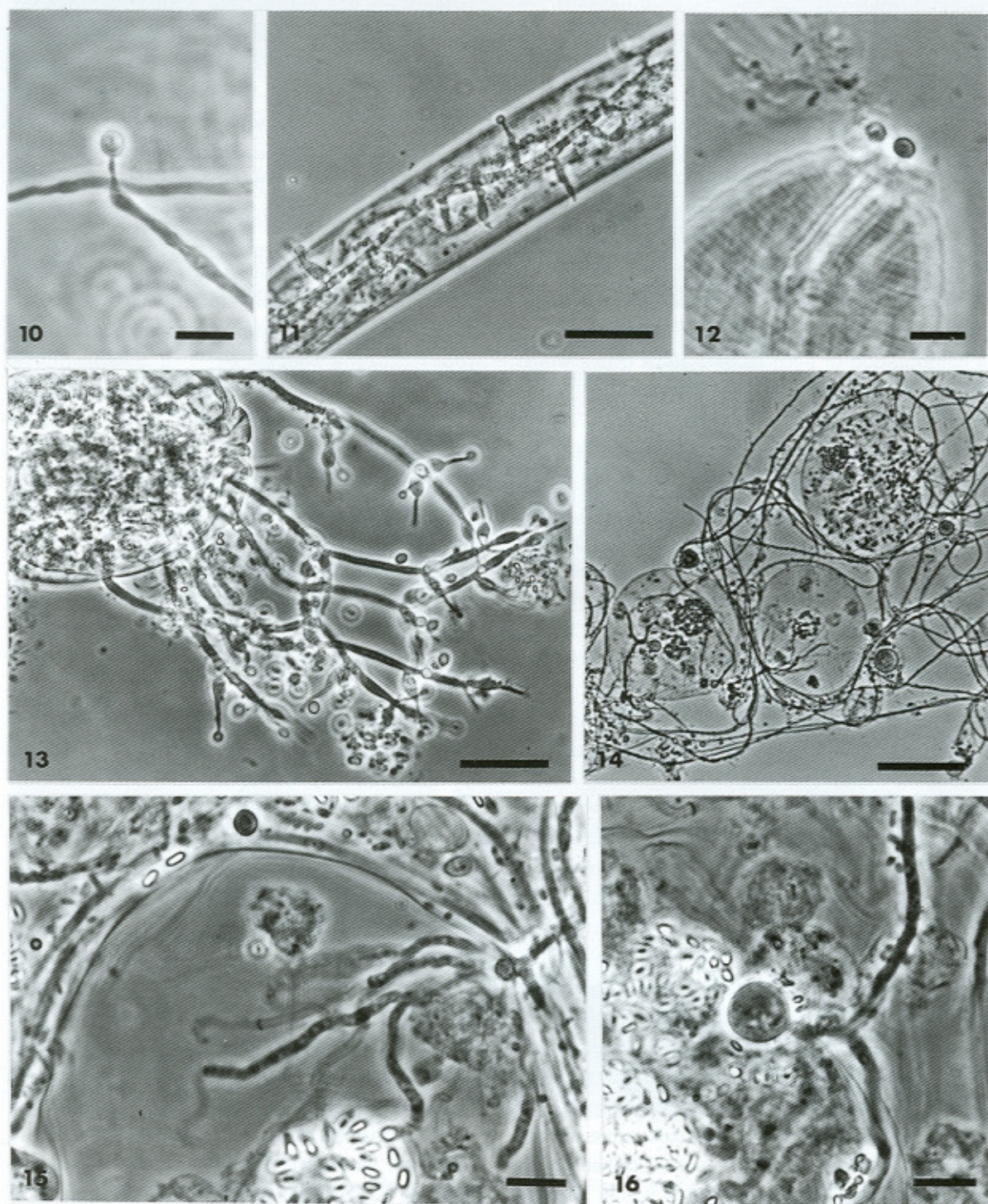


Fig 10 Spherical conidium from culture of *Plesiospora globosa* (Scale bar, 6  $\mu$ m). Fig 11 Nematode infected by *Tolypocladium lignicola* (Scale bar, 20  $\mu$ m). Fig 12 Conidia of *T. lignicola* at the mouth of a nematode (Scale bar, 6  $\mu$ m). Fig 13 Bdelloid rotifer infected with *Rotiferophthora* (Scale bar, 20  $\mu$ m). Fig 14 Amoebae trapped by mycelium of zoopagalean fungus (Scale bar, 40  $\mu$ m). Fig 15 Assimilative hyphae of zoopagalean fungus inside amoeba (Scale bar, 6  $\mu$ m). Fig 16 Developing zygospore of zoopagalean fungus (Scale bar, 6  $\mu$ m).

described (Glockling & Shimazu, 1997) and were later grown on CMA and incubated at 25°C.

#### Results and discussion

The Amami rabbit dung was found to harbour an

interesting diversity of predacious, endoparasitic and parasitoidal fungi and a total of 16 species were isolated during this survey. These included five species of predacious hyphomycetous fungi which capture nematodes in traps. Also in the

Fungi Imperfecti, six species endoparasitic in nematodes and one species endoparasitic in rotifers were found. Within the Zygomycotina, one member of the Zoopagales capturing amoebae was found, and an entomophthoraceous fungus which was parasitic in tardigrades. One species of zoosporic oomycetous fungi holocarpic in nematodes was found in the rabbit dung. One surprising result from the survey was the presence of *Dactylella leptospora* Drech. (Drechsler, 1937) and *Dactylaria candida* (Nees.) Sacc. (Saccardo, 1886) which capture nematodes with a combination of both passive rings (rings which do not constrict), and adhesive stalked knobs (Fig 2). There are only three species known which produce this combination of trapping apparatus. After capture of a nematode by an adhesive knob or a passive ring, the fungus forms a large infection bulb inside the body which effectively severs the internal organs and kills the nematode (Fig 3). The adhesive knobs are easily detached from the hyphae after contact with a nematode and they penetrate into the nematode body and produce assimilative hyphae (Fig 4). The passive rings are also commonly detached as they are held on narrow hyphae which are easily broken if a strong nematode is captured. The nematode swims away with the rings around its body (Fig 5) and is later infected. The ease with which the captured nematodes break the hyphal supports of the trapping devices ensures dispersal of the fungus away from the mycelium. Another fungus producing only stalked knobs, *Dactylella ellipsozona* Grove (Grove, 1885) was also isolated from the rabbit dung. Modern techniques (Borrebaek *et al.*, 1984) have shown that nematode-trapping structures produce carbohydrate-binding proteins which bind to N-acetyl-D-galactosamine on the nematode surface. In this way, the stalked knobs are host-specific for nematodes which secrete substances which bind them to the knobs, and are unable to capture nematodes which do not secrete the chemicals needed for adhesion. When Drechsler described *Dactylella leptospora* (Drechsler, 1937) he reported that the stalked knobs in his cultures were ineffective and that only the passive rings were able to capture nematodes. The existence of the chemical bond between the nematode hosts and the adhesive knobs was not known at that time and it is now evident that the nematodes in Drechsler's cul-

tures were not compatible with *D. leptospora*. Friman (1993) developed a method for isolating the adhesive knobs of *Dactylaria candida* so that future studies of the trap-specific microbodies will be possible. Conidia of both *Dactylella leptospora* and *Dactylaria candida* showed an ability to germinate to produce adhesive knobs directly. Species which display this ability for early trap development were thought to exhibit less saprophytic ability and more host-dependence but, as previously stated (Cooke, 1963), no study to assess the extent to which these nematode-trapping species exist in a saprophytic capacity in the wild has ever been made, and all are able to grow with varying growth rates on artificial media.

The two *Arthrotrichs* species isolated during this survey, *A. cladodes* Drech. (Drechsler, 1937) and *A. superba* Corda (Corda, 1839) are fairly well-known species which are commonly reported from a variety of habitats. Neither of these species produced chlamydospores in culture and both produced conidia with a single septum just below or near the centre. The isolate of *A. cladodes* Drech. agreed well with that of the original description. The fungus identified as *A. superba* Corda had both proliferating and branching conidiophores but differed slightly from Drechsler's isolate in having slightly broader conidia. Conidia ranged from a short, squat form to a cylindrical, elongate form. Although both types were produced on one conidiophore, they were borne in separate whorls. A closer identification for this isolate could not be found. Both these fungi and *Dactylaria thaumasia* Drech. (Drechsler, 1937) construct three-dimensional networks of adhesive hyphal loops in which nematodes become entangled.

The endoparasitic hyphomycetes found included three species of *Harposporium*, one species of *Rotiferophthora*, and one species of *Verticillium*. Also, a rare fungus belonging to a monotypic genus, *Plesiospora*, was found infecting just two nematodes. In addition, a *Tolyocladium* species originally described from bdelloid rotifers and producing small disc-shaped conidia, was isolated from a single nematode host. *Harposporium* and *Rotiferophthora* species usually infect the host with infection conidia which are ingested and which germinate inside the host. All the nematodes infected by *Verticillium balanoides* (Drech.) Dowsett *et al.*

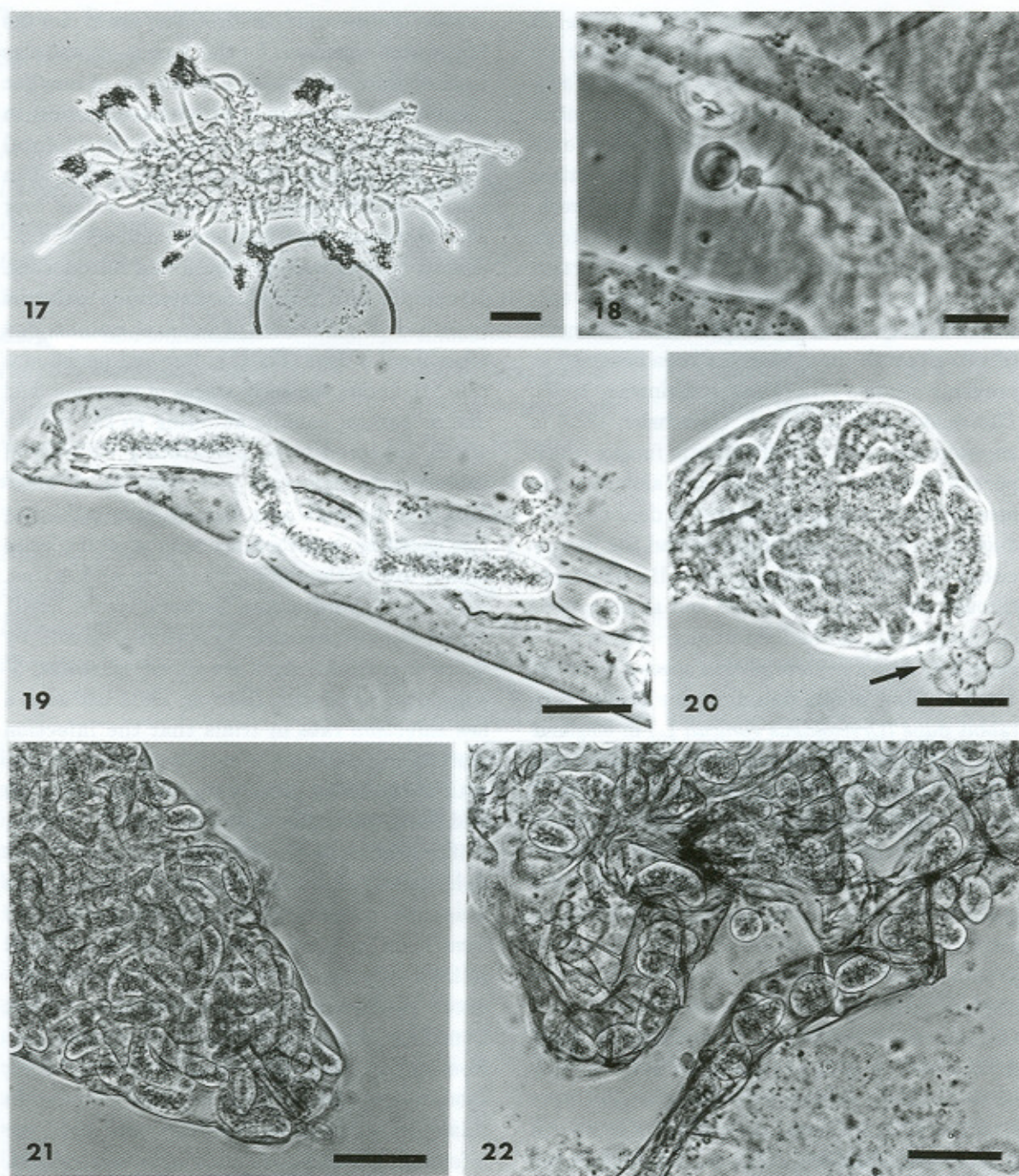


Fig 17 Tardigrade infected by *Ballocephala sphaerospora* (Scale bar, 5  $\mu$ m). Fig 18 Infection conidium of *B. sphaerospora* penetrating into tardigrade body (Scale bar, 6  $\mu$ m). Fig 19 *Myzocytiopsis* in nematode host (Scale bar, 20  $\mu$ m). Fig 20 *Myzocytiopsis* in rotifer with empty zoospore cysts on cuticle (arrow) (Scale bar, 6  $\mu$ m). Fig 21 *Myzocytiopsis* in tardigrade (Scale bar, 20  $\mu$ m). Fig 22 *Myzocytiopsis* in mite (Scale bar, 20  $\mu$ m).

(Drechsler, 1941; Dowsett *et al.*, 1982) (Fig 6) were *Plectus* species which often burrow under the agar surface in Petri dish cultures. Infected specimens were commonly under the agar and long conidiophores of *V. balanoides* grew from the submerged body and eventually through the agar into the air. The fungus was able to sporulate under the agar as well as aerially but aerial

sporulation was more profuse. *V. balanoides* has acorn-shaped conidia (Fig 7) which are adhesive at the distal end and which stick to the nematode cuticle (Fig 7, arrow) and penetrate it with a germ tube to initiate infection. *Harposporium anguillulae* Lohde (Lohde, 1874) was the first endoparasitic nematophagous fungus to be described and also the first species to be cul-

tured artificially on agar medium (Aschner & Kohn, 1958). *H. anguillulae* and *H. leptospira* are common endoparasites found in a variety of substrates. These fungi both produce curved infection conidia and also form chlamydo-spores inside the nematode body. The other *Harposporium* species found, *H. cycloides* Drech. (Drechsler, 1968), produced curved conidia from the infected nematode (Fig 8). In pure culture, in addition to sharply pointed curved conidia, this strain of *H. cycloides* also produced arthroconidia (Fig 9) although these have not been observed in previous studies (Glockling & Shimazu, 1997). *Plesiospora globosa* Drech. (Drechsler, 1970) is an unusual and rare endoparasitic fungus which parasitises a species of slender, slow-moving nematode. It is unusual because it does not produce any external conidiophores and the narrow necks of the conidiogenous cells protrude through the nematode cuticle and small spherical conidia are formed externally. There have been few, if any, reports of this species since its discovery but it was found on one occasion in rabbit dung (*Oryctolagus cuniculi* L.) in England infecting a single nematode (Glockling, unpublished thesis data). On the previous occasion, *P. globosa* was not isolated into culture but this time a pure culture was successfully obtained from one of the two infected specimens. This is the first time that this fungus has been grown on artificial media. In culture on CMA, *P. globosa* produced small spherical conidia from flask-shaped and elongate conidiogenous cells (Fig 10). Conidia were produced in abundance and often formed in long chains in a similar mode to that of *Verticillium coccosporum* (Drechsler) Gams (Drechsler 1941; Gams, 1988) which also infects nematodes. Reinfection experiments to decipher the mode of infection for *P. globosa* were not carried out at this time as the host nematode for this fungus has not yet been identified. The other nematophagous endoparasitic species isolated, *Tolypocladium lignicola* Barron (Barron, 1983), also appears to be an uncommon fungus. It was initially isolated from rotten wood using baiting techniques with rotifers although, during reinfection experiments, Barron noted that rotifers ingested, digested and excreted large numbers of *T. lignicola* conidia, apparently using them as a food source. Only the occasional conidium lodged in the rotifer mastax region and germinated to produce infection. It may be that

species of nematodes, not rotifers, are in fact the target host organisms for this fungus. It was also isolated from nematodes in a sample of muntjac (*Muntiacus*) dung on a previous occasion in England (Glockling, unpublished thesis data) when nematodes were repeatedly infected in mixed culture containing bdelloid rotifers over several weeks. On CMA large numbers of disc-shaped conidia were produced and introduction of conidia into *Rhabditis* nematode cultures resulted in infection of several nematodes after about 4 days (Fig 13). Infected nematodes (Fig 11), during the early stages of infection, had one or several conidia adhering to the mouth which appeared to be penetrating via a germ tube into the nematode body (Fig 12). If infection of *T. lignicola* is in fact by spore adhesion, this would be in accordance with several species which are pathogenic to insects and mites. However, *T. parasiticum* Barron (Barron, 1980), which is endoparasitic in rotifers, infects by spore ingestion. Another rotifer parasite, *T. trigonosporum* Barron (Barron, 1981), had such a low infection rate in trials that the infection method could not be ascertained. For species with a capability of infecting both nematodes and rotifers, the infection method may differ according to the host organism. The *Rotiferophthora* species (Fig 13) infected rotifers in samples collected at the same time in summer from both Naon and Kawauchi. Many bdelloid rotifers succumbed to the infection which produced an abundance of small infection conidia from flask-shaped conidiogenous cells and also from aphanophialides. Multi-septate resting spores (dictyochlamydo-spores) were produced in this species but they were very rare until the infection, which persisted over several weeks, began to decline. Specimens infected during the end of the infection duration produced up to three dictyochlamydo-spores which became large and bullate at maturity. The *Rotiferophthora* superficially resembled *R. turbinaspora* Barron (Barron, 1985) and *R. barronii* Glockling (Glockling & Dick, 1994) but differed from these species in the size and shape of the conidiogenous cells and conidia. Consequently, this species is being described as new (Glockling, in preparation).

The zoopagalean fungus which trapped amoebae on adhesive aseptate hyphae (Fig 14) was recovered from Amami rabbit dung from two col-

Table 1 Fungi isolated from Amami rabbit dung.

Species isolated	Host	Trap/Infection method
<i>Arthrotrrys superba</i>	Nematode	Adhesive network
<i>Arthrotrrys cladodes</i>	Nematode	Adhesive network
<i>Dactylaria thaumasia</i>	Nematode	Adhesive network
<i>Dactylaria candida</i>	Nematode	Passive ring/stalked knob
<i>Dactylella leptospora</i>	Nematode	Passive ring/stalked knob
<i>Dactylella ellipsospora</i>	Nematode	Stalked knob
<i>Harposporium anguillulae</i>	<i>Rhabditis</i> nematode	Spore ingestion
<i>Harposporium leptospira</i>	<i>Rhabditis</i> nematode	Spore ingestion
<i>Harposporium cycloides</i>	<i>Rhabditis</i> nematode	Spore ingestion
<i>Verticillium balanoides</i>	<i>Plectus</i> nematode	Spore adhesion
<i>Plesiospora globosa</i>	Nematode	?
<i>Tolypocladium lignicola</i>	Nematode	Spore adhesion?
<i>Rotiferophthora</i> sp.	Bdelloid rotifer	Spore ingestion
Zoopagalean sp.	Amoeba	Adhesive hyphae
<i>Ballocephala sphaerospora</i>	Tardigrade sp.	Spore adhesion
<i>Myzocytiopsis</i> sp.	<i>Rhabditis</i> nematode	Zoospore encystment

lections taken in winter and summer. The fungus did not produce conidia on either occasion so it could not be identified to species level but the morphology of the assimilative hyphae was similar to that of *Stylopage areae* Drech. (Drechsler, 1935) and *Zoopage mitospora* Drech. (Drechsler, 1938) although zygospores have not been described for either of these species. At the point of attachment and penetration of the fungus into the amoeba host, the fungus produced several assimilative branches (Fig 15). Spherical zygospores were formed inside the captured amoebae after intertwining of two hyphae (Fig 16).

The tardigrade parasite, *Ballocephala sphaerospora* Drech. (Drechsler, 1951), produced short conidiophores from the tardigrade body and masses of infection conidia were formed from short, swollen conidiogenous cells (Fig 17). Later, resting spores developed inside the body. Infection was initiated by small spherical spores which attached to the host cuticle and penetrated with a fine germ tube (Fig 18). The species of tardigrade infected in the Amami rabbit dung appeared similar to the *Macrobotus* species infected in Drechsler's original isolate. There are only three known species of *Ballocephala*, all infecting tardigrades. None of these zygomycete

fungi have been cultured at this time.

The *Myzocytiopsis* fungus, which produced motile biflagellate zoospores, infected *Rhabditis* nematodes in several cultures derived from rabbit dung collected from Naon and Kawachi. The fungus formed swollen cylindrical zoosporangia which developed evacuation tubes to release the zoospores (Fig 19). Rotifers were not often infected but one specimen (Fig 20) was found to have thalli of *Myzocytiopsis* in the egg inside the rotifer body, and empty zoospore cysts were still attached to the rotifer cuticle (arrow). Zoospores emerged from the evacuation tubes of the zoosporangia initially as an amoeboid mass, but individual zoospores became apparent after a period of slow movement. Tardigrades were also occasionally infected by the fungus (Fig 21) and one mite also succumbed to infection (Fig 22). This is the first record of a mite being attacked by *Myzocytiopsis*.

### Conclusions

A diversity of common and rare fungi and one new species were found in the Amami rabbit dung. The occurrence of several host-specific endoparasitic and predacious fungi as well as those with a broad host range reflected the wide diversity of host organisms which had colonized

the dung habitat. Rarely reported species of endoparasitic fungi are usually associated with the rarer nematode species which thrive within a narrow range of conditions and which are not so fecund as common species. The presence of *Dactylaria candida* and *Rotiferophthora* in cultures prepared from collections taken from both Kawauchi and Naon during the same collection trip showed that there was effective local dispersal over 10 km. The Amami rabbit dung proved to be a good source of predacious fungi, especially those capturing nematodes on adhesive knobs.

The importance of dung as a habitat for fungi which attack microscopic animals has not yet been thoroughly investigated, but it would appear that a rabbit pellet provides a choice temporary habitat for several transitory host-specific fungi as well as those with greater tolerance.

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