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Zombie bugs? The fungus *Purpureocillium* cf. *lilacinum* may manipulate the behavior of its host bug *Edessa rufomarginata*

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Abstract: Just before dying, *Edessa rufomarginata* (Hemiptera, Pentatomidae) individuals that are infected with the fungus *Purpureocillium* cf. *lilacinum* (Ascomycota: Ophiocordycipitaceae) move from the leaves onto the stems of their *Solanum* sp. host and firmly grasp the stems in ways seldom employed by uninfected bugs. These alterations in host behavior probably improve the chances that the subsequently produced fungal spores will be dispersed aerially. *Purpureocillium* cf. *lilacinum* is a member of the Ophiocordycipitaceae, a group in which other species also modify the behavior of their hosts. As in the case of newly distinguished relatives of *Ophiocordyceps unilateralis* associated with “zombie ants” the discovery of *P.* cf. *lilacinum* infecting bugs reveals that *P. lilacinum* may be more diverse than previously appreciated.

Key words: Costa Rica, insect, parasitic manipulation, pentatomid bug

INTRODUCTION

Some parasitic organisms, including fungi, alter or manipulate the behavior of their hosts in ways that promote the survival and reproduction of the parasite (summaries in Libersat et al. 2009, Hughes et al. 2012). Several groups of entomopathogenic fungi induce their hosts to occupy a position that is thought to be advantageous for spore dispersal. Some zygomycetes in Entomophthorales induce insects to move upward on plants. Just before killing the host fly, *Scathophaga stercoraria*, *Entomophthora muscae* induces the fly to perch at unusual sites on emergent vegetation in open fields in atypical ways: higher above the ground than usual, at the tips of leaves or flowers on the downwind side of the plant, facing inward toward the plant, grasping the leaf or flower with its legs, and lowering its wings and elevating its abdomen from which the spores will emerge (Maitland 1994). Another entomophthoralean fungus may induce a related host fly, *Musca domestica*, to raise its abdomen, raise its wings and extend its mouthparts to the substrate, allowing a strong attachment to form (Brobyn and Wilding 1983, Krasnoff et al. 1995). Such modifications probably increase the release and dispersal of the spores when they are shed from the fly.

Similar behavior by “zombie” ants is caused by certain ascomycetes (Evans et al. 2011). Some members of *Ophiocordyceps* (= *Cordyceps*) spp. (Hypocreales, Ophiocordycipitaceae) (Sung et al. 2007) induce infected worker ants to seize a twig, a leaf or another object firmly with their legs and mandibles (Pontoppidan et al. 2009, Evans et al. 2010, Evans 2012). Infected workers of the ant *Camponotus leonardi*, the principal host of *Ophiocordyceps unilateralis*, grasp the undersides of leaves just before dying (Pontoppidan et al. 2009). Ascospores escape from perithecia embedded in an elongated stroma on the dorsum of the ant, and they are thought to be dispersed more successfully than if the ant had stayed within its nest and been dumped onto the refuse pile within its nest or had simply fallen to the ground after death (Hughes 2012). The biting behavior of the ants and their tendency to be in plants above the forest floor are aberrant behaviors induced by the infection;

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uninfected ants do not normally hold leaves with their mandibles. Biting the substrate maintains the ant in situ after it dies, giving the fungus time to produce an adhesive pad that more permanently binds the ant's body to the plant (Pontoppidan et al. 2009).

This report presents observations suggesting that the fungus *Purpureocillium* cf. *lilacinum* modifies the behavior of the pentatomid bug *Edessa rufomarginata*. Other strains of *P. lilacinum* have been isolated from soil, air and animals, including immunocompromised humans (Saghroun et al. 2013), nematodes and various insects, such as curculionid beetles, homopterans and lepidopteran larvae (Spatafora et al. 2007), and from a related bug, *Edessa meditabunda* (Humber 2007); one species of *Ophiocordyceps*, *O. pentatoma* (Koval) (Mycobank MB504321) was isolated from a pentatomid bug in Russia (Sung et al. 2007b). Possible effects on the host behavior were not noted. The bug in the present study, *Ed. rufomarginata*, is a large (about 1.5–1.8 cm long), widespread, herbivorous Neotropical pentatomid found on plants in several families (Silva et al. 2004, Silva and Oliveira 2010). Some species of this subfamily are pests of cultivated plants (Silva and Oliveira 2010).

MATERIALS AND METHODS

Field observations.—They were made during the day near Silencio (elevation approximately 50 m, near the Rio Savegre), Puntarenas province, Costa Rica (–84.0236W, 9.4165N) on 14–17 Jan 2013 and 20 Jan. 2014 along the edges and in the interior of plantations of African oil palms (*Elais guineensis*). Although this was the dry season, substantial rains occurred nearly every afternoon and at night. All observations of adult *Edessa rufomarginata* were of individuals on the leaves or the stems of *Solanum* sp., an early succession weed that prefers well illuminated sites, where its erect and semirecumbent stems reach about 1.5 m. We did not search for bugs on the ground below plants, where they would have been difficult to find in the tangled vegetation and could well have been carried off by ants. Both adults and nymphs of the bugs were present, but no dead nymphs were observed. A few bugs (< 5) also were seen on a second species of *Solanum* but are not included here. Specimens of the bugs with and without fungus are deposited in the Museo de Zoología of the Escuela de Biología of the Universidad de Costa Rica.

We noted the positions of all living and dead adult bugs that we found. No plant was checked more than once, so individual bugs were probably never observed more than once. We noted the sites on the plant where bugs occurred (leaf, petiole, stem) and the position of each leg: whether the tarsi or the tibiae made contact with the plant; and (for bugs on stems or petioles) whether any legs were flexed so that the tarsus or tibia crossed the bug's midline ventrally (scored as “embraced the stem” in the descriptions below). Two further details were noted for a subset of bugs: whether

the legs contacted the plant on the tips of the trichomes and spines that covered the stem or on the surface of the stem itself; and whether the bug's mouthparts were inserted into the plant (with the protective labrum folded posteriorly). Sample sizes are not equal for all legs; in some cases it was either not possible to check all legs without disturbing the bug or some legs were missing.

The presence of fungus was confirmed in two dead bugs found on the host plant that did not show visible fungal growth externally, by dissecting one (which proved to be tightly packed with fungal hyphae) and by keeping the other in a humid environment for 2 d, after which fungal hyphae emerged from its membranes. We use the term “infected” in the descriptions below to indicate dead bugs with fungus. We noted no differences between male and female bugs and combine them in the descriptions. We kept several infected bugs in closed humid chambers for up to 2 wk after collection and noted changes in the morphology of the fungal growth on them.

Fungal isolation.—Spores from four dead individuals of *E. rufomarginata* collected at Silencio, Rio Savegre, Puntarenas, Costa Rica, were transferred from the surface of the exoskeleton to fresh yeast extract (YM) agar plates (0.5% yeast extract, 0.3% malt extract, 2% agar) using sterile technique. After 3 d incubation at room temperature, the fungal isolates were purified by multiple subculturing and stored on YM agar plates and broth at –80 C in 15% glycerol (Mueller et al. 2004). Cultures were deposited in the collections of the Herbarium of the Escuela de Biología, Universidad de Costa Rica (accession number USJ 83667), and the CBS Fungal Diversity Centre, Utecht, the Netherlands (accession number 2670) as *Purpureocillium* sp. (isolates 1–4).

Amplification and sequencing of DNA.—Genomic DNA was extracted from the cultures with a Wizard® Genomic DNA purification kit (Promega). The concentration and integrity of total extracted DNA were confirmed by gel electrophoresis in 0.8% agarose in 0.5× Tris-Borate-EDTA (TBE) buffer. Rapid identification was carried out by PCR amplification and sequencing of ~ 500 bp of the internal transcribed spacers 1 and 2 (ITS) recommend as a barcode marker for fungi (Schoch et al. 2012). The amplification was carried out with primers ITS1 (forward) (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (reverse) (5'–TCCTCCGCTTATTGATATGC–3') and the PCR protocol recommended by White et al. 1990. The purified PCR products were sequenced in both directions by Beckman Coulter Genomics (Danvers, Massachusetts) and submitted to GenBank (TABLE I).

Phylogenetic analyses.—Contig sequence and sequencing manipulations were carried out using Se-AL 2.01a11 (<http://tree.bio.ed.ac.uk/software/seal/>) and MESQUITE (Maddison and Maddison 2010). The chromatograms were corrected by eye and the poor quality edges were trimmed. The sequences (TABLE I) were aligned in the online interface MAFFT 6.859 (<http://mafft.cbrc.jp/alignment/software/>) with advanced alignment strategy with one conserved domain (L-INS-i). All sequences not listed individually in the tree were grouped as *Purpureocillium*

TABLE I. GenBank accession numbers of the ITS sequences used in the phylogenetic analysis. Sequences in boldface were obtained in this study

GenBank	Species	GenBank	Species
AB103380	<i>P. lilacinum</i>	HQ829095	<i>P. lilacinum</i>
AF368804	<i>P. lilacinum</i>	HQ842812	<i>P. lilacinum</i>
AM412779	<i>P. lilacinum</i>	HQ842813	<i>P. lilacinum</i>
AY213666	<i>P. lilacinum</i>	HQ842814	<i>P. lilacinum</i>
AY213668	<i>P. lilacinum</i>	HQ842815	<i>P. lilacinum</i>
AY624189	<i>P. lilacinum</i> ^T	HQ842816	<i>P. lilacinum</i>
DQ187953	<i>P. lilacinum</i>	HQ842818	<i>P. lilacinum</i>
DQ641505	<i>P. lilacinum</i>	HQ842819	<i>P. lilacinum</i>
EU553282	<i>P. lilacinum</i>	HQ842821	<i>P. lilacinum</i>
EU553290	<i>P. lilacinum</i>	HQ842823	<i>P. lilacinum</i>
EU553316	<i>P. lilacinum</i>	HQ842824	<i>P. lilacinum</i>
EU553319	<i>P. lilacinum</i>	HQ842825	<i>P. lilacinum</i>
EU553336	<i>P. lilacinum</i>	HQ842826	<i>P. lilacinum</i>
EU828665	<i>P. lilacinum</i>	HQ842827	<i>P. lilacinum</i>
FJ461773	<i>P. lilacinum</i>	HQ842829	<i>P. lilacinum</i>
FJ765019	<i>P. lilacinum</i>	HQ842832	<i>P. lilacinum</i>
FJ765020	<i>P. lilacinum</i>	HQ842833	<i>P. lilacinum</i>
FJ765021	<i>P. lilacinum</i>	HQ842834	<i>P. lilacinum</i>
FJ765022	<i>P. lilacinum</i>	HQ842835	<i>P. lilacinum</i>
FJ877138	<i>P. lilacinum</i>	HQ842836	<i>P. lilacinum</i>
FJ904282	<i>P. lilacinum</i>	HQ842837	<i>P. lilacinum</i>
FJ904283	<i>P. lilacinum</i>	HQ842838	<i>P. lilacinum</i>
FJ973075	<i>P. lilacinum</i>	JF824690	<i>P. lilacinum</i>
FN598940	<i>P. lilacinum</i>	JF896084	<i>P. lilacinum</i>
FR822391	<i>P. lilacinum</i>	JF896085	<i>P. lilacinum</i>
GQ229072	<i>P. lilacinum</i>	JF896086	<i>P. lilacinum</i>
GQ229079	<i>P. lilacinum</i>	JN650588	<i>P. lilacinum</i>
GQ229080	<i>P. lilacinum</i>	JN850995	<i>P. lilacinum</i>
GQ229083	<i>P. lilacinum</i>	JN851054	<i>P. lilacinum</i>
GQ241282	<i>P. lilacinum</i>	JQ627630	<i>P. lilacinum</i>
GQ376101	<i>P. lilacinum</i>	JQ763398	<i>P. lilacinum</i>
GU130296	<i>P. lilacinum</i>	JQ781830	<i>P. lilacinum</i>
GU453928	<i>P. lilacinum</i>	JQ863231	<i>P. lilacinum</i>
GU453929	<i>P. lilacinum</i>	JQ866689	<i>P. lilacinum</i>
GU980015	<i>P. lilacinum</i>	JQ866690	<i>P. lilacinum</i>
GU980017	<i>P. lilacinum</i>	JX969622	<i>P. lilacinum</i>
GU980020	<i>P. lilacinum</i>	JX978452	<i>P. lilacinum</i>
GU980023	<i>P. lilacinum</i>	KC254065	<i>P. lilacinum</i>
GU980024	<i>P. lilacinum</i>	KC311490	<i>P. lilacinum</i>
GU980026	<i>P. lilacinum</i>	KC524426	<i>P. lilacinum</i>
GU980027	<i>P. lilacinum</i>	KC551953	<i>P. lilacinum</i>
GU980030	<i>P. lilacinum</i>	KC551961	<i>P. lilacinum</i>
GU980031	<i>P. lilacinum</i>	HE792981	<i>P. lavendulum</i>
GU980039	<i>P. lilacinum</i>	FR734106	<i>P. lavendulum</i> ^T
HM032028	<i>P. lilacinum</i>	FR734107	<i>P. lavendulum</i>
HM242263	<i>P. lilacinum</i>	EU086434	<i>Haptocillium balanoides</i>
HM242264	<i>P. lilacinum</i>	AJ292419	<i>Haptocillium zeosporum</i>
HM439952	<i>P. lilacinum</i>	KJ577794	<i>P. cf. lilacinum</i> isolate 1 on <i>Edessa</i>
HQ607796	<i>P. lilacinum</i>	KJ577795	<i>P. cf. lilacinum</i> isolate 2 on <i>Edessa</i>
HQ647313	<i>P. lilacinum</i>	KJ577796	<i>P. cf. lilacinum</i> isolate 3 on <i>Edessa</i>
HQ829056	<i>P. lilacinum</i>	KJ577798	<i>P. cf. lilacinum</i> isolate 4 on <i>Edessa</i>

^T Type species.

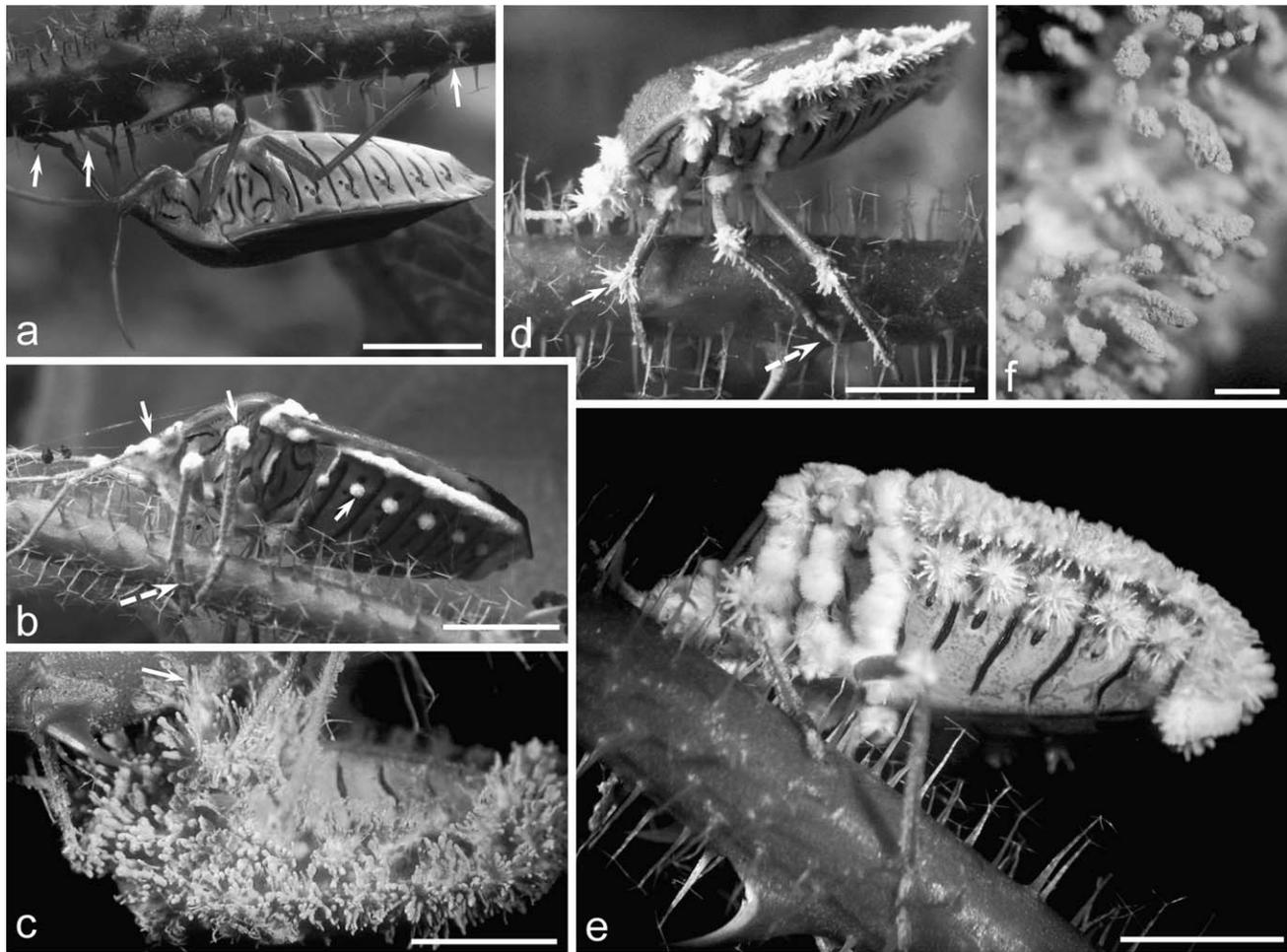


FIG. 1. *Edessa rufomarginata* bugs (Pentatomidae, Hemiptera) and *P. cf. lilacinum* (Ophiostomataceae). a. A living uninfected bug on a stem, showing the tarsi contacting the plant (arrows); the legs are not embracing the plant. b. An early stage of fungal growth with white mycelium visible at antennal and leg articulations and abdominal spiracles (arrows). One leg (III) is embracing the stem (dotted arrow) (note also the antennae deflected ventrally compare with a). c. A late stage of fungus infection. The bug is covered with hyphae and developing synnemata with gray conidia at maturity; the hyphae extend and adhere (solid arrow) to the plant stem. d. An early intermediate growth stage with mycelium protruding from the leg joints with synnemata developing (solid arrow). One leg (II) is embracing the stem. e. Late intermediate stage in which the fungus has begun to form synnemata and has conidia darkening at some sites (e.g. the abdominal spiracles), an indication of conidial maturity; sites where hyphae emerged later (e.g. the thoracic pleura) are still white. f. Higher magnification of synnemata at a late stage of growth.

lilacinum s.s. Maximum likelihood (ML) phylogenetic inference was performed in RaxML 7.2.6 (Stamatakis 2006) using a partitioned dataset (partitions for ITS1, 5.8S, ITS2) under a general time reversible model with a gamma distribution of site rate variation (GTRGAMMA) with ML support estimated with 1000 bootstrap replicates. Alignments and trees were deposited in TreeBASE (accession number S15527). Tree editing was done with FigTree 1.3.1 software (<http://tree.bio.ed.ac.uk/software/figtree/>).

RESULTS AND DISCUSSION

Fungal infection.—Of the 256 bugs we observed in 2013 (FIG. 1a–e), 15 (5.9%) were infected. Of the 241

living bugs, 149 (61.8%) were on leaves, 84 (34.9%), on stems and 8 (3.3%) on petioles. Mating pairs were seen on both leaves and stems. Of the 15 infected bugs in 2013 and seven additional infected individuals found in 2014, 19 (86.4%) were on stems, 2 (9.1%) were on a petiole and 1 (4.5%) embraced the tip of a leaf ($\text{Chi}^2 = 27.0$, $\text{df} = 1$, $P < 0.001$ comparing homogeneity of proportions of living and infected bugs on leaves versus rounded structures that the bug could embrace) (stems and petioles). Among 73 living bugs that were checked for feeding while on stems, 30 (41.1%) had their mouthparts inserted into the plant; in contrast none of the 17 infected bugs in

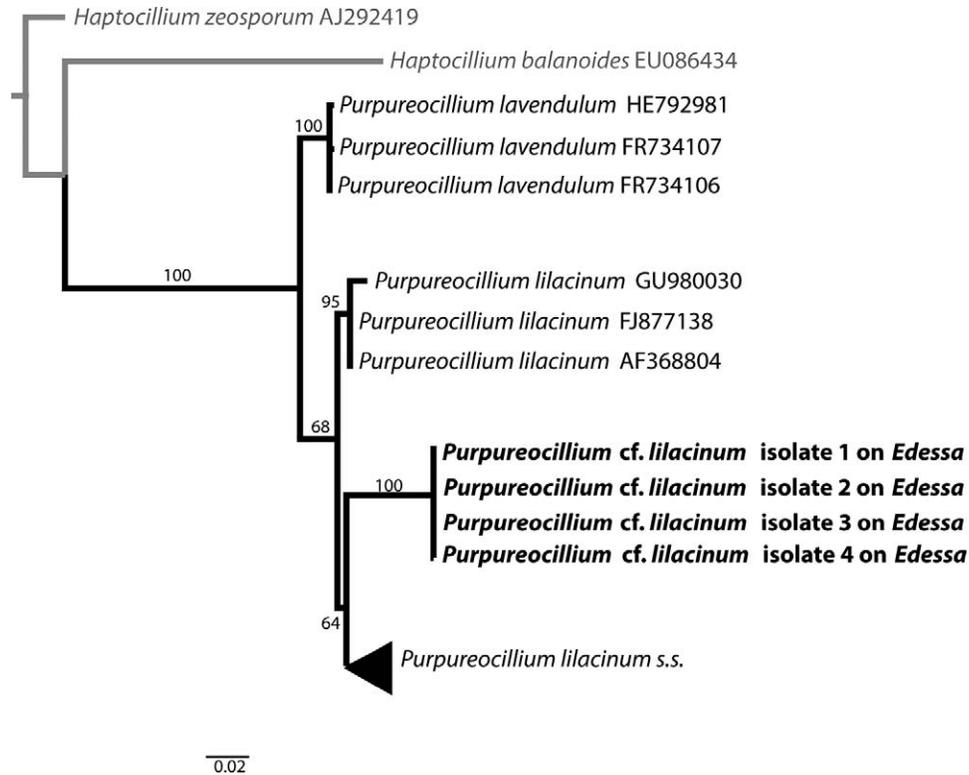


FIG. 2. Consensus ML tree based on 106 ITS sequences of *Purpureocillium* spp. using a dataset of 528 characters. Outgroups are denoted with branches in gray. The final ML optimization likelihood is 1600.36.

which the mouthparts were visible (e.g. not covered by fungal growth) had their mouthparts inserted into the plant ($\text{Chi}^2 = 10.5$, $\text{df} = 1$, $P = 0.0012$). When feeding bugs were disturbed, they often tugged against their mouthparts, which seemed to anchor them briefly to the plant. Bugs on leaves were not checked for mouthpart insertion; they were difficult to observe for this detail in that they moved readily when approached, suggesting that they were usually not feeding.

Of the 84 living bugs on stems whose leg positions were noted, only three individuals had even a single leg embracing the stem; in total, only three of 300 legs (1.0%) that were scored embraced the stem. The corresponding number for infected bugs was 21 of 116 legs (18.1%) in 22 bugs ($\text{Chi}^2 = 45.0$, $\text{df} = 1$, $P < 0.001$) (FIG. 1b, d). Thirteen of 22 infected bugs (59.1%) embraced with at least one leg, as compared with only three of 84 living bugs (3.6%) ($\text{Chi}^2 = 45.8$, $\text{df} = 1$, $P < 0.0001$).

Fine white fungal growth first emerged from membranes between segments of dead bugs (e.g. between leg and antennal segments and somewhat later from the spiracles and at the lateral edges of the abdominal tergites (FIG. 1b, d). Later white mats of fungus emerged along the lateral edges of the bug's abdomen and other parts of its body (FIG. 1c, e) and

synnemata developed from the mycelium (FIG. 1f); different parts of the same bug sometimes had different stages of fungal growth (FIG. 1e). In a few cases the fungus reached the surface of the plant stem where it adhered (FIG. 1c). As the fungus matured and covered the bug more completely, it appeared gray rather than white (FIG. 1c), due to conidium maturation. A gray cloud of spores floated in the air when a bug with mature growth (FIG. 1c) was jarred. Observations in the field and in humid chambers in captivity indicated that the progression of the fungal infection occurred over the space of several days; we estimate that older infected bugs in the field probably been dead for a week or more.

Both the tendency of infected bugs to rest on stems and to embrace them anchored the bug more firmly to the plant and probably reduced the chances that it would fall to the ground. This could aid in dispersing the dry, aerial fungal spores during the long period of sporulation. These changes in bug behavior would qualify as manipulation, because they are likely to be advantageous for the fungus.

Observations of living bugs indicate that feeding bugs were physically anchored to the plant by their mouthparts. Nevertheless, unlike the ants infected with the related fungus *Ophiocordyceps unilateralis* and the *Musca* flies infected with *Entomophthora muscae*

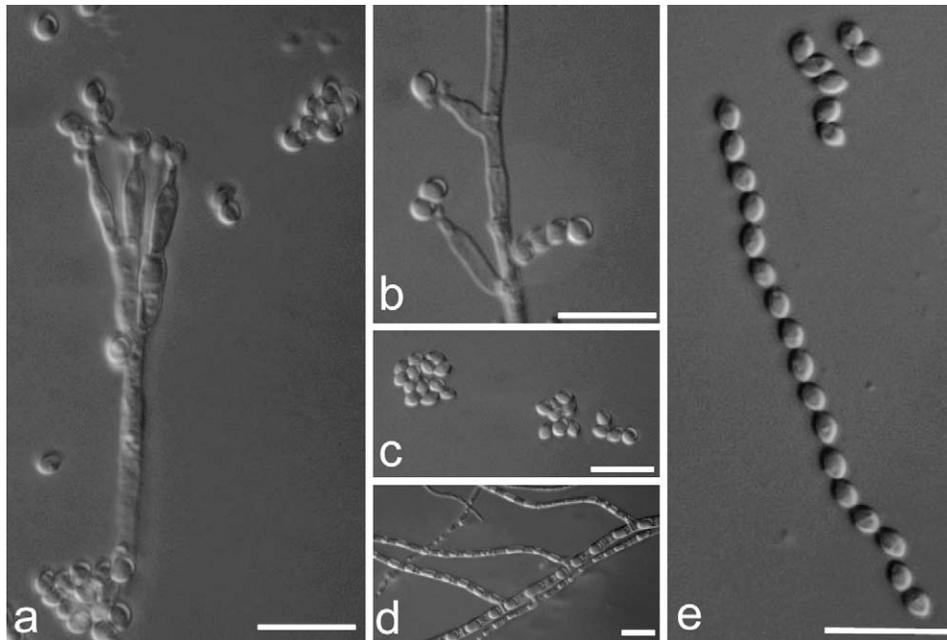


FIG. 3. *Purpureocillium* sp. on YM agar after 3 d. a. Verticillate conidiophores and chains of ovoid conidia. b. *Acremonium*-like conidiophores and chains of ovoid conidia. c. Detached ovoid conidia. d. Hyphae with abundant lipid droplets. e. Chain of conidia. Bars = 10 μ m.

fungus, the infected bugs whose mouthparts were checked did not have their mouthparts extended or inserted into the plant. Thus the fungus *P.* cf. *lilacinum* differs from these other fungi in failing to affect the host's use of its mouthparts, even though insertion of the mouthparts would have provided a further anchor to the plant.

Our data may overestimate the relative frequency with which infected bugs died on stems rather than leaves. If bugs were killed while they were on leaves but then fell to the ground, we would have missed them. Nevertheless our substantial sample of dead infected bugs, the nearly total absence of dead infected bugs on leaves and the absence of dead bugs without fungi on either stems or leaves make it likely that the fungus biases the site where the bug will die in favor of stems and increases the likelihood that it will embrace the stem just before death.

Our interpretation that the fungus manipulates the bug's behavior depends on the assumption that the fungal infection precedes the bug's death, an assumption that needs to be tested in further studies. In addition, we observed living bugs only during the day. We cannot rule out the possibility that they may frequently be located on stems rather than leaves more frequently during the night. If this were true (we have no evidence one way or the other) and if the bugs died at night but not during the day (again we have no evidence), the bias to die on stems rather than leaves could be achieved by killing the bugs at

night without actively influencing the bug's behavior. Nevertheless the additional manipulation we documented regarding the positions of the bug's legs, especially embracing the stem, cannot be easily explained in this way.

Fungal characterization.—Based on evidence from morphological and molecular characters, the pure cultures obtained from the four *E. rufomarginata* specimens are identical strains of *P.* cf. *lilacinum* (FIGS. 1–3b, c, d, e). The colonies were pinkish-lilaceous on YM media, and verticillate conidiogenous structures arose from hyphae to produce ovoid conidia (FIG. 3a); other dry conidia were produced in sympodial succession from *Acremonium*-like conidiophores (FIG. 3b), distinguishing this strain from other members of the species. Neither synnemata production nor spore germination at 37 C was observed in culture. Based on a BLAST query of sequences in GenBank, 22 nucleotide differences separated the ITS sequences of *P.* cf. *lilacinum* and sequences of the closest strains, all identified as *P. lilacinum* (Luangsa-ard et al. 2011).

Only two species of *Purpureocillium* have been described, *P. lilacinum* (the type species of the genus) (Luangsa-ard et al. 2011) and *P. lavendulum* (Perdomo et al. 2013). Many insect- and nematode-associated isolates of *P. lilacinum* have been reported from widespread localities in the Americas, Europe and Asia, both in the literature (Luangsa-ard et al.

2011, Perdomo et al. 2013) and in the USDA-ARS Collection of Entomopathogenic Fungal Cultures (ARSEF) (Humber 2007). Although our strains were nested among other strains identified as *P. lilacinum*, differences among the strains, including habitat, geographical distribution and especially the ITS sequence divergence indicate that *P. lilacinum* is more diverse than is presently recognized. Unfortunately, because of permit problems and time constraints, we were unable to obtain DNA from key cultures to test this hypothesis and we have not described new species in this report.

While much work remains to document evolution of behavioral manipulation by different parasitic groups, one possible emerging pattern is the following. The more distantly related zygomycete (*Entomophthora muscae*) produces behavioral modifications that in some respects resemble more closely the effects produced by *P. cf. lilacinum* (perching on certain parts of plants, embracing the plant with the legs) than do those of the much more closely related *Ophiocordyceps* spp. despite the likely independent evolutionary origins of these effects. This evolutionary flexibility in manipulation, which exploits host behavior and natural history, echoes a similar pattern in ichneumonid polysphinctine wasps that manipulate the web construction behavior of their web-building spider hosts (Gauld and Dubois 2006, Korenko et al. 2013). The phylogeny of these parasites also seems not to be a good predictor of the effects that they have on their hosts' behavior (Eberhard 2013). The mechanisms of host manipulation remain to be determined in both wasps and fungi, but the behavioral details they elicit do not appear to be especially constrained by phylogeny in either group.

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