Mycologia, 96(5), 2004, pp. 955–959.

Pathogenicity of *Escovopsis weberi*: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus

Hannah T. Reynolds[1]
Cameron R. Currie[2]

Department of Ecology and Evolutionary Biology, 1200 Sunnyside Ave., University of Kansas, Lawrence, Kansas 66045

Abstract: Fungi in the genus *Escovopsis* are known only from the fungus gardens of attine ants. Previous work has established that these anamorphic fungi, allied with the Hypocreales, are specialized and potentially virulent parasites of the ancient mutualism between attine ants and their fungal cultivars. It is unclear whether the primary nutrient source for the pathogen is the mutualist fungal cultivar or the vegetative substrate placed on the gardens by the ants. Here, we determine whether *Escovopsis weberi* is a parasite of the fungal cultivar, a competitor for the leaf substrate, or both. Bioassays reveal that *E. weberi* exhibits rapid growth on pure cultivar and negligible growth on sterilized leaf fragments. Light microscopy examination of hyphal-hyphal interactions between *E. weberi* and the ant fungal cultivar indicate that *E. weberi,* unlike invasive necrotrophs that always penetrate host hyphae, can secrete compounds that break down host mycelium before contact occurs. Thus, *E. weberi* is a necrotrophic parasite of the fungal cultivar of attine ants.

Key words: *Acromyrmex, Atta,* fungus-growing ants, mutualism, mycoparasite, necrotroph

INTRODUCTION

Leaf-cutter ants, members of the tribe Attini, cultivate fungi in the family Lepiotaceae (Basidiomycota), which serve as their main food supply (Hölldobler and Wilson 1990, Chapela et al 1994). They provide fresh leaves and flowers as substrate to support the growth of the cultivar. The ants also promote the growth of their mutualistic fungus through pruning and the movement of enzymes within the garden (Martin 1970, 1987, Martin and Martin 1970, Bass and Cherrett 1996). They defend their fungal cultivar from invading microbes by grooming and licking the fungus garden and by removing (weeding) infected parts of the garden (Stahel and Giejskes 1939, Autuori 1941, Quinlan and Cherrett 1977, 1979, Currie and Stuart 2001).

Escovopsis is a genus of anamorphic fungi allied with the ascomycetous order Hypocreales (Currie et al 2003). It is commonly associated with the garden of fungus-growing ants, including the leaf-cutters, having been isolated in more than 50% of the gardens sampled in some studies (Currie et al 1999, Currie 2001b). Fungi in this genus appear to be obligately specialized on the attine ant-microbe symbiosis; they have been found growing naturally only in ant fungal gardens and associated dumps (Seifert et al 1995, Currie et al 1999, Bot et al 2001). Previous work has established that *Escovopsis* is a parasite of this mutualism (Currie et al 1999, Currie 2001b). The microfungus can limit the fitness of a colony and devastate a garden in a few days (Currie et al 1999; Currie 2001b, 2001a). *Escovopsis* also fulfills Koch's postulates of pathogenicity; when it was isolated from diseased gardens and reapplied to healthy gardens, it caused the same disease (Currie et al 1999). *Escovopsis,* which does not produce airborne spores and apparently is not transmitted by founder queens, might be vectored by other invertebrates living in association with ant colonies (Currie et al 1999, Currie 2001a). In addition, it is an ancient member of the attine ant-microbe symbiosis, having co-evolved with the ants and their cultivar (Currie et al 2003).

While *Escovopsis* is clearly a parasite of the fungus garden, the precise nature of its pathogenicity is unknown. It might be a parasite of the cultivar, gaining nutrients from the mycelium of the basidiomycete; on the other hand, it might be a ''weed'', competing for the substrate placed on the gardens by the ants (Currie 2001a). In this study, we examined the question of whether *E. weberi* is a parasite of the cultivar of leaf-cutter ant gardens, a saprobe competing for the leaf substrate, or both. We used microscopy techniques and growth studies to determine *E. weberi*'s methods of mycoparasitism.

MATERIALS AND METHODS

Seven colonies of leaf-cutter ants (three *Atta colombica* and four *Acromyrmex octospinosus*) collected from Panama in 2001 were used in these experiments (collection numbers:

Accepted for publication March 25, 2004.
[1] E-mail: htfreynolds@hotmail.com
[2] Corresponding author. E-mail: ccurrie@bact.wisc.edu

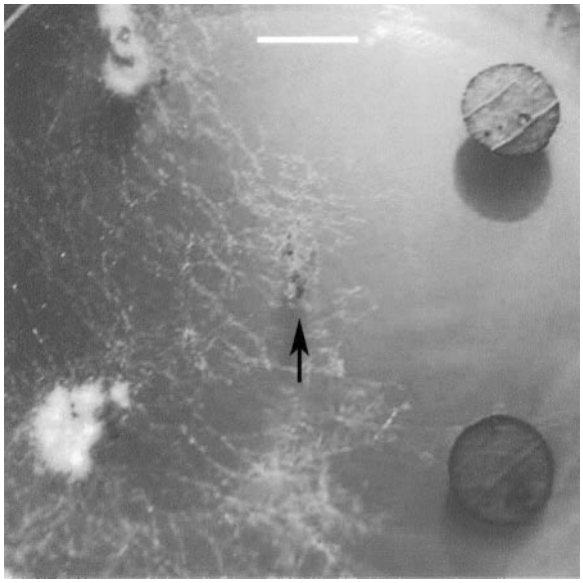

FIG. 1. Culture growth of *E. weberi* in the presence of leaves and the fungal cultivar of leaf-cutter ants. *E. weberi* was inoculated in the center of the plate (indicated by black arrow) and grew first toward cultivar pieces, then covered the entire plate. In this picture, *E. weberi* hyphae are clearly covering the half of the plate containing the two cultivar pieces and are almost in contact with the bottom leaf piece. Scale bar = 3 mm.

CC010327-07, CC011010-04, CC011213-16, CC011213-29, CC011213-35, SP011017-04, and SP011017-05). The colonies were maintained in the laboratory in plastic polystyrene boxes (10⅜ × 7$^{9}/_{16}$ × 3¾ inches) placed on plastic polystyrene box ''islands'' (7⅜ × 5¼ × 3¼ inches). These ''islands'' were kept on trays (21¼ × 10½ × 2½ inches) filled with soapy water to prevent movement of mites that could vector *E. weberi* spores among colonies.

To examine the ability of *E. weberi* (Muchovej and Della Lucia 1990) to grow on leaves and cultivar, we conducted bioassays on four different substrates, as well as on a nutrient-free control treatment. The five treatments were: (i) four pieces of the axenic cultivar (c), (ii) two pieces of the cultivar + two pieces of leaves manually cut (ccl), (iii) four pieces of manually cut leaves (cl), (iv) four pieces of leaves taken from ant colonies (al), and (v) no nutrient source added (control). All bioassays were conducted in 60 × 15 mm Petri plates on water agar (15 g agar/L water). The bottom of each plate was marked using a square stencil (2.1 × 2.1 cm). The substrate pieces were placed outside the corners of the square, and *E. weberi* was inoculated aseptically in the center of each plate (see FIG. 1). Five plates of each treatment were made to test *E. weberi* strains from each of the seven colonies, for a total of 175 plates (7 *E. weberi* isolates × 5 treatments × 5 replicates/treatment = 175). Isolates of the fungal cultivar and *E. weberi* were obtained by plating garden pieces on potato-dextrose agar (PDA) plates (see Currie et al 1999). *E. weberi* was tested against the cultivar from the garden from which it was isolated. Cultures of the fungi used in the experiments are stored at the National Museum of Natural History, Smithsonian Institution, under the aforementioned collection numbers. The leaves used for both the manual cutting treatment as well as the ant-cut leaves were from Redbud (*Cercis canadensis*). Manual cutting was done with a 3 mm cork borer, and the leaf fragments then were autoclaved. Leaf material taken from ant colonies was collected from the surface of fungus gardens, once the ants had masticated it. It was washed with alcohol and water to remove garden fungus and autoclaved. Although the leaf fragments were at the top of the garden and had not been fully integrated into the fungal matrix, visible cultivar hyphae were in the area, making it necessary to sterilize the leaf surfaces to ensure that, if *E. weberi* grew toward the leaves, it was not attracted to latent cultivar fragments. After surface sterilization, the leaves were autoclaved. To perform our experiments, we found it necessary to fully sterilize the leaves; surface sterilized leaves that were not autoclaved became overgrown with several types of fungi overnight when placed on water agar. Digital photographs were taken of each plate daily for 1 wk with a Nikon Coolpix 5000 camera. The photographs were examined to determine when *E. weberi* hyphae initially reached the pieces of substrate or the equivalent distance in the case of the control plates. Growth was quantified as the number of substrate pieces reached. Some plates became contaminated and thus were not used in the analyses. Because of contamination, the treatments each had different numbers of total substrate pieces. The numbers of plates that could be scored were: *Acromyrmex octospinosus* c = 15, ccl = 13, al = 13, cl = 14, control = 15; *Atta colombica* c = 16, ccl = 19, al = 17, cl = 12, control = 20, for a total of 154 plates and 616 substrate pieces. The data were analyzed using Chi-square tests (Steel and Torrie 1980).

To establish the method of mycoparasitism, the hyphal interactions between *E. weberi* and the cultivar were examined under light microscopy (Nikon Eclipse E600). Nineteen slide cultures were made using 3 × 1 × 1 mm slides and squares of PDA. Cultivar was inoculated onto PDA and allowed to grow into the medium for 1 wk. *E. weberi* then was inoculated onto the same PDA at the other end of the slide, and the slide was covered with a cover slip. The slides were photographed daily for 1 wk after inoculation with *E. weberi* and then reviewed to observe any fading of the cultivar hyphae before the first contact with *E. weberi* hyphae.

RESULTS

E. weberi grew preferentially toward the cultivar pieces (FIGS. 1 and 2), causing them to turn yellow or brown and to shrivel. The parasite grew poorly in the absence of the cultivar, even when leaves were present. There was significantly higher growth in the presence of the cultivar than in the presence of leaves alone ($\chi^2 = 335.611$, $P < 0.001$). When *E. weberi* was presented both with leaves and with cultivar, it typically grew toward the cultivar first and then spread over the rest of the plate (FIG. 1). There was no significant difference in the amount of growth of *E. we-*

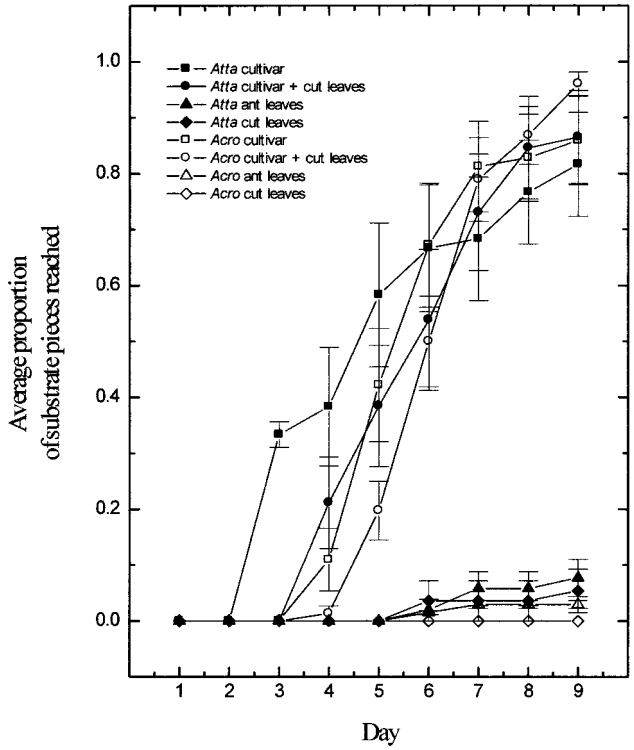

FIG. 2. *E. weberi* growth toward leaves and leaf-cutter ant fungal cultivar. The graph displays the growth of *E. weberi* from *Atta colombica* and *Acromyrmex octospinosus* colonies during 1 wk. Y-axis data were generated by examining digital photographs to determine how many substrate pieces were in contact with *E. weberi* on any given day.

beri from *Atta colombica* colonies and from *Acromyrmex octospinosus* colonies ($\chi^2 = 1.124$, $P = 0.289$). No growth of *E. weberi* was observed in the water-agar control treatments.

In 11 slide cultures, the leading cultivar hyphae were broken down before contact by *E. weberi* hyphae. The breakdown of the cultivar hyphae was characterized by a loss of opacity, which was observed throughout the cultivar mycelium, and not only in the leading edge, although the leading hyphae were most affected (FIG. 3). In seven of the slide cultures, the cultivar hyphae were not noticeably faded before contact with *E. weberi*. One of the slides became contaminated before contact between the two fungi could occur.

DISCUSSION

E. weberi is clearly capable of obtaining the nutrients it requires for growth from the leaf-cutter ant cultivar. It grew extremely well with the cultivar as its only source of nutrients in Petri plate bioassays in this study and in broth bioassays (Reynolds unpubl data). In contrast, *E. weberi* exhibited minimal or no growth on both cut leaves and ant-treated leaves. The specialized niche of *E. weberi* in the gardens of fungus-growing ants also indicates that it is not a weed; if it were a saprotroph, using leaf material as substrate for growth, it might be expected to occur outside this mutualism. *E. weberi* typically is found growing in the bottom of gardens, where the nutritive value of the leaves has already been reduced by the cultivar fungus (Currie 2001b), further suggesting that it is not a competitor for the leaf material. Thus, our results indicate that *E. weberi* is a parasite of the fungal cultivar and not a weed.

There are two basic types of mycoparasites: biotrophs, which feed on the living host cytoplasm, and necrotrophs, which kill their hosts, then digest the dead biomass (Jeffries and Young 1994). The discoloration and collapse of the cultivar mycelium in this experiment and the collapse of the ant gardens in experiments by Currie et al (1999) demonstrate that *E. weberi* kills the cultivar to gain nutrients from it and is therefore a necrotroph. Using light microscopy techniques, we observed that *E. weberi* in many cases was able to degrade cultivar hyphae from a distance of at least 25 μm; penetration was not necessary for parasitism to occur. We concluded that *E. weberi* is a contact necrotroph that does not have to penetrate the hyphae of its host, as opposed to an invasive necrotroph that must penetrate host tissue (Jeffries and Young 1994).

Our finding that *E. weberi* is a necrotrophic parasite is not surprising perhaps because it is closely related to other necrotrophic parasites. The sister group of *Escovopsis* is the family Hypocreaceae (Currie et al 2003b), which includes many mycoparasitic fungi. For example, several species of *Hypomyces* are parasites of fungi in the family Boletaceae (Rogerson and Samuels 1989). The genus *Trichoderma,* an anamorph allied with the Hypocreaceae, includes ecologically dominant necrotrophic species, some of which parasitize the mushroom *Agaricus bisporus* (Jeffries and Young 1994, Castle et al 1998, Samuels et al 2002). *Trichoderma* sp. are contact necrotrophs capable of using several types of antagonism: (i) long-range enzyme activity (Dennis and Webster 1971), (ii) hyphal interference involving close-range toxin (volatile and nonvolatile) production and coiling around host hyphae, and (iii) penetration (De-Oliveira et al 1984, Jeffries and Young 1994). Perhaps *Escovopsis* spp., like *Trichoderma* spp., are capable of multiple types of antagonism; further work must be done to determine whether it is capable of coiling and/or penetration.

The fungal genus *Escovopsis* is a group of highly evolved obligate mycoparasites. It has an ancient origin within the attine ant-microbe mutualism and ap-

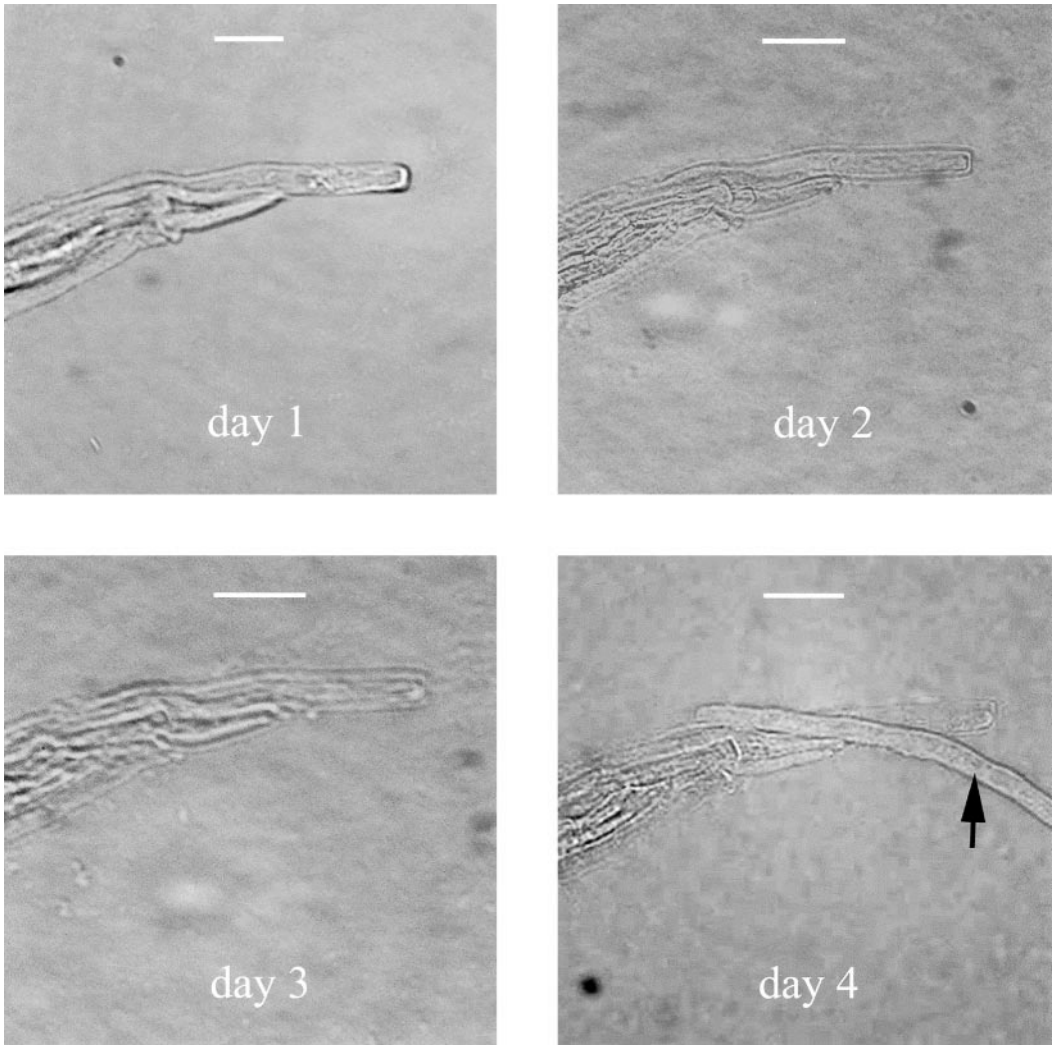

FIG. 3. Slide culture of a bioassay between leaf-cutter ant fungal cultivar and *E. weberi*. *E. weberi* was inoculated to the right of the displayed cultivar hyphae. Cultivar hyphae are clearly visible on Day 1. On days 2 and 3, the hyphae have lost opacity and the cell walls are more difficult to see. On Day 4, the cultivar hyphae are further faded, and an *E. weberi* hypha (see black arrow) has crossed over them. Each scale bar = 10 μm.

parently has been co-evolving with the ants and their cultivar for more than 50 000 000 yr (Currie et al 2003). In this study, we established that *E. weberi* acts as a necrotrophic parasite on the cultivar of leaf-cutter ants, the most phylogenetically derived genera of fungus-growing ants. Future work should examine the activity of species of *Escovopsis* that infect the gardens of the more basal ant genera. Indeed, studies on the pathogenicity of *Escovopsis* spp. across the extant phylogenetic diversity of this ancient and host-specific association could lead to better understanding of the evolution of mycoparasitism in general. In addition, further studies on the mechanisms of *Escovopsis* pathogenicity might provide insights for using the fungus in biological control efforts for leaf-cutter ants, which are economically important pests in the Neotropics (Weber 1972).

ACKNOWLEDGMENTS

We would like to thank Matías Cafaro, Robert Lichtwardt, Ainslie Little, Keith Seifert, Merlin White and an anonymous reviewer for valuable suggestions on this manuscript and Anne Danielson-François, Emily Davenport, María Leone, Lacey Loudermilk, Emily Magee, Shauna Price and Alison Stuart for logistical support. This research was supported by grants from NSF (Integrative Research Challenges in Environmental Biology DEB-0110073) and from the University of Kansas Honors Program. We also thank STRI, Autoridad Nacional del Ambiente of the Republic of Panama for facilitating research and granting collecting permits.

LITERATURE CITED

Autuori M. 1941. Contribuicao para o conhecimento da sauva (*Atta* spp.) I. Evolucao do sauveiro (*Atta sexdens*

rubropilosa Forel, 1908). Arq Inst Biol Sao Paulo 12: 197–228.

Bass M, Cherrett JM. 1996. Leaf-cutting ants (Formicidae, Attini) prune their fungus to increase and direct its productivity. Funct Ecol 10:55–61.

Bot ANM, Currie CR, Hart AG, Boomsma JJ. 2001. Waste management in leaf-cutting ants. Ethol Ecol Evol 13: 225–237.

Castle A, Speranzini D, Rghei N, Alm G, Rinker D, Bissett J. 1998. Morphological and molecular identification of *Trichoderma* isolates on North American mushroom farms. Appl Environ Microbiol 64:133–137.

Chapela IH, Rehner SA, Schultz TR, Mueller UG. 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science 266:1691–1695.

Currie CR, Mueller UG, Malloch D. 1999. The agricultural pathology of ant fungus gardens. Proc Natl Acad Sci USA 96:7998–8002.

———. 2001a. A community of ants, fungi and bacteria: a multilateral approach to studying symbiosis. Annu Rev Microbiol 55:357–380.

———. 2001b. Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia 128:99–106.

———, Stuart AE. 2001. Weeding and grooming of pathogens in agriculture by ants. Proc R Soc Lond 268:1033–1039.

———, Wong B, Stuart AE, Schultz TR, Rehner SA, Mueller UG, Sung G, Spatafora JW, Straus NA. 2003. Ancient tripartite coevolution in the attine ant-microbe symbiosis. Science 299:386–388.

Dennis C, Webster J. 1971. Antagonistic properties of species groups of *Trichoderma*. Trans Br Mycol Soc 57:41–48.

DeOliveira VL, Bellei M, Borges AC. 1984. Control of white rot of garlic by antagonistic fungi under controlled environmental conditions. Canadian Journal of Microbiology 30:884–889.

Hölldobler B, Wilson EO. 1990. The ants. Cambridge, Massachusetts: Belknap.

Jeffries P, Young TWK. 1994. Interfungal parasitic relationships. Wallingford, Oxon UK: CAB International.

Martin MM. 1970. The biochemical basis of the fungus-attine ant symbiosis. Science 169:16–20.

———. 1987. Invertebrate-Microbial Interactions. Ithaca, NY: Cornell Univ. Press.

———, Martin JS. 1970. The biochemical basis for the symbiosis between the ant, *Atta colombica tonsiper* and its food fungus. J Insect Phys 16:109–119.

Muchovej JJ, Della Lucia TMC. 1990. *Escovopsis,* a new genus from leaf-cutting ant nests to replace *Phialocladus* nomen invalidum. Mycotaxon 37:191–195.

Quinlan RJ, Cherrett JM. 1977. The role of substrate preparation in the symbiosis between the leaf-cutting ant *Acromyrmex octospinosus* (Reich) and its food fungus. Ecol Entomol 2:161–170.

———, ———. 1979. The role of the fungus in the diet of the leaf-cutting ant *Atta cephalotes*. Ecol Entomol 4: 151–160.

Rogerson CT, Samuels GJ. 1989. Boleticolous species of *Hypomyces*. Mycologia 81:413–432.

Samuels GJ, Dodd SL, Gams W, Castlebury LA, Petrini O. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. Mycologia 94:146–170.

Seifert KA, Samson RA, Chapela IH. 1995. *Escovopsis aspergilloides,* a rediscovered hyphomycete from leaf-cutting ant nests. Mycologia 87:407–413.

Stahel G, Geijskes DC. 1939. Ueber den Bau der Nester von *Atta cephalotes* (L.) und *Atta sexdens* (L.) (Hym: Formicidae). Revista Ent Rio J 10:27–78

Steel RGD, Torrie JH. 1980. Principles and procedures of statistics: a biometrical approach. 2nd ed. New York: McGraw-Hill Publishing Co.

Weber NA. 1972. Gardening ants, the attines. Philadelphia, Pennsylvania: American Philosophical Society.