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IDENTIFICATION OF THE ETIOLOGICAL AGENT FOR NECROTIZING SCUTE DISEASE IN THE TEXAS TORTOISE

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ABSTRACT: Epidermal lamellae (scutes) of the Texas tortoise, *Gopherus berlandieri*, from southern Texas (USA) were observed to be in various stages of necrosis, ranging from localized whitish blemishes to complete degradation of the external portion of the scute. *Fusarium semitectum* was consistently isolated from slivers of infected scute from tortoises. The fungus was not isolated from tortoises exhibiting no lesions. Confocal microscopy confirmed the presence of septate mycelia inside the scutes, and isolates of *F. semitectum* grown in the laboratory were successfully transferred to non-infected tortoises. Twenty-four tortoises maintained by two rehabilitators in southern Texas exhibited lesions; however, only one of 27 tortoises from Dimmit and Zavala counties was infected.

Key words: *Fusarium semitectum, Gopherus berlandieri,* keratin, scute disease, Texas tortoise.

INTRODUCTION

One of us (FLR) has studied the general biology of the Texas tortoise (*Gopherus berlandieri*) for over 30 yr (Judd and Rose, 2000). Over this time, numerous tortoises were noted to have whitish blemishes associated with the keratinaceous epidermal scutes (epidermal lamellae) that overlaid the bony elements of the carapace and plastron. On occasion, the blemishes coalesced and encompassed large sections of the carapace and plastron, such that areas of the tortoise appeared whitish.

Growth annuli as registered in the horny scutes of *G. berlandieri* are distinctly ridged (Ernst et al., 1994) but the ridges become less distinct with age because of wear. On occasion, however, individuals or shells were found on which the scutes were smoothed as though polished. Individual scutes in this condition are translucent and, on occasion, the underlying bone can be seen.

In 1995, we obtained a tortoise shell exhibiting scutes in various stages of necrosis (Fig. 1), and in 1996, we received a living tortoise with extensive white blemishes (Fig. 2) and which exhibited scutes in various stages of necrosis. We believe that the two observed conditions are the result of the same disease at various stages in its ontogeny and that a fungus (*Fusarium semitectum*) is the etiological agent.

METHODS AND MATERIALS

General

Gopherus berlandieri is listed as threatened by the state of Texas (USA) (Rose and Judd, 1982). Confiscated or injured animals are maintained at Southwest Texas State University (SWT; San Marcos, Texas) and by various approved rehabilitators. We cultured slivers of infected scute from six *G. berlandieri* maintained at SWT, three maintained at Laguna Vista (Texas; 26.101°N, 97.290°W) and nine maintained at Donna (26.170°N, 98.052°W). In addition, shells of individuals from Hidalgo (9) (26.100°N, 98.263°W), Cameron (6) (25.901°N, 97.497°W) and Dimmit (28.522°N, 99.860°W) and Zavala (28.677°N, 99.828°W) counties (27) were available for examination.

Isolation of the fungus

The presumed infected scute area to be exercised was scrubbed thoroughly with bactericidal soap, cleansed with hydrogen peroxide (1.5%), towel dried, and removed with a sterile razor blade. Care was exercised in selecting areas of excision that were free of external manifestation of the disease, i.e., whitish lesions were visible within the scute but no external damage was evidenced. Small slivers of scute were placed in keratin agar plates containing 5% bovine keratin (ICN Pharmaceuticals, Inc., Costa Mesa, California, USA) suspended in sterile distilled water and solidified with 3% agar. No other nutrients were added so that contamination of non-keratinaceous airborne fungi would be limited or prohibited. After 7 to 10 days incubation at room temperature the cultures were examined for fungal growth.

Slivers of scutes were removed from six tor-

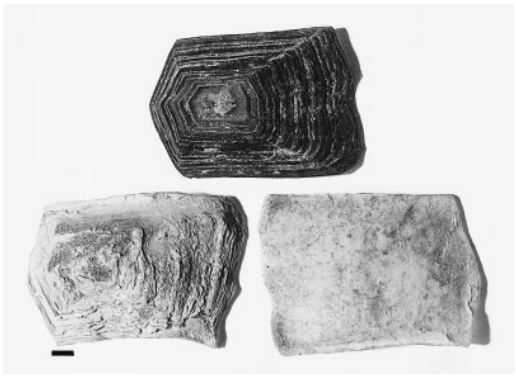

FIGURE 1. Scutes from three different adult *Gopherus berlandieri.* The top scute is normal the left scute is diseased and the right is advanced but showing no evidence of continued keratin involvement. Bar = 10 mm.

toises that were received from a rehabilitator in Harris County (Texas; 30.097°N, 95.616°W) and which showed no evidence of fungal involvement. The slivers were treated as were those from infected tortoises.

Slivers of presumed infected scutes that exhibited no external manifestation of the fungus were washed with antibacterial soap, dipped in 3% hydrogen peroxide, patted dry, and placed in 10% KOH to dissolve the keratin. Hyphae released from the interior of the scute via this process were stained with Trypan blue to enhance their visibility.

Identification of the fungus

The fungus growing on the keratin-enhanced agar was identified as *Fusarium semitectum,* based on its morphology, by D. Sutton (Fungus Testing Laboratory, University of Texas Health Science Center, San Antonio, Texas). Identification was verified by staff at the Fusarium Research Center (Pennsylvania State University, University Park, Pennsylvania, USA). The identification based on DNA sequence analyses of ribosomal RNA gene spacer sequences was compared to known species of *Fusarium* by E. Cigelnik and K. O'Donnell (National Center for Agricultural Utilization Research, Peoria, Illinois, USA).

Microscopic examination of the fungus

Isolated *F. semitectum* from tortoise scute was examined using differential interference

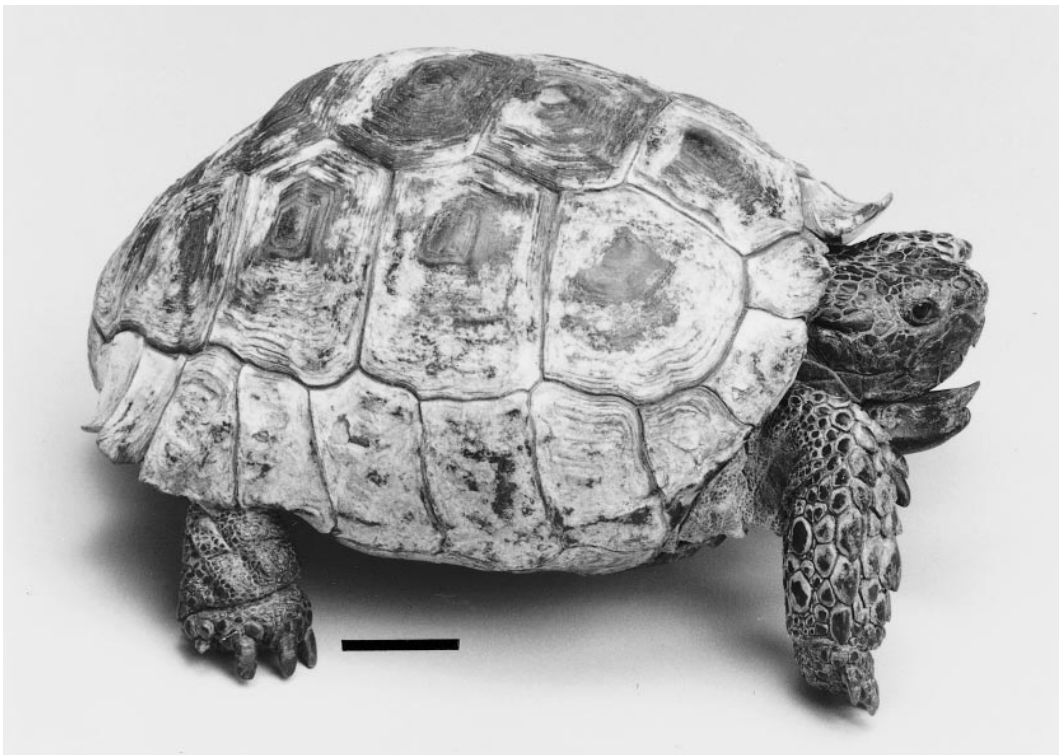

FIGURE 2. Adult male *Gopherus berlandieri* exhibiting extensive white lesions attributed to keratin degradation by *Fusarium semitectum.* Bar = 10 mm.

contrast (DIC) microscopy. DIC images were obtained and recorded using the free software, NIH Image (http://rsh.info.nih.gov/nih-image.)

To verify the presence of hyphae within the scute, infected tortoise scute fragments were sliced tangentially and mounted on microscope slides with coverslips. Hyphae were revealed within the scute by detection of chitin autofluorescence against the less fluorescent background of the scute (primarily keratin). Multiple optical sections were obtained creating a data array that allowed determination of image elements (hyphae) with respect to other structures in three dimensions. Fluorescence excitation was achieved using the green line of a krypton-argon laser (λ = 514 nm) (Bio-Rad 1024 laser scanning confocal microscope, Hercules, California, USA) and emitted light of 600 nm and above was collected from multiple image planes. To convert the image to the gray scale shown here, the color information was removed and the intensity inverted to produce a black on white micrograph.

This technique allowed us, using the same image data set, to rotate the plan view 90° clockwise, creating a lateral view of the specimen. Microscopy was performed on an Olympus IX-70 (Olympus, Melville, New York) inverted microscope coupled to a Bio-Rad MRC 1024 Laser. Confocal image acquisition and initial processing were done using Bio-Rad's Lasersharp software running on a Compaq PC (Compaq, Houston, Texas). Final processing and printing of images was done using Adobe Photoshop software (Adobe, San Jose, California) running on a G3 Macintosh (Apple, Cupertino, California) and driving an Epson Stylus 850 color ink-jet printer (Epson, Portland, Oregon, USA).

For scanning electron microscope imaging a scute sample was fixed in 4% paraformaldehyde, dehydrated by critical point drying, sputter coated with gold, and examined and photographed using a Cambridge 90B scanning electron microscope (Cambridge Instruments, Oxford, UK).

Host inoculation

Capped centrifuge tubes (1.5 ml capacity) (Baxter Scientific Products, McGraw Park, Illinois, USA) were cut in half and glued using epoxy resin onto the scutes creating a cup-like chamber that could be accessed via the cap. Each chamber provided two cm^2 of scute surface. Individuals were chosen that showed no manifestation of the disease. Mycelia harvested from hyphae grown in keratin enriched agar were mixed with sterile water at 8×10^5 cells/ml. Three *G. berlandieri* were fitted with six tubes each, three (experimentals) which received 200 μl of the fungal suspension. Three tubes (controls) received 200 μl of sterile water. Selection of the sites was random to mitigate position effects.

Seven box turtles (*Terrapene ornata*) were fitted with two control and two experimental cups and treated as were the tortoises. Similar chambers were glued onto box turtles, which were chosen because they were available and the disease has never been observed by us on any member of that genus. Box turtle chambers were inoculated similarly to those on the tortoises.

RESULTS

Fungal mycelia observed on tortoise shells and in petri dishes appeared powdery and white to cream in color. Differential interference contrast microscopy revealed a fungus with hyaline septate hyphae, a phialide with a collarette visible at the apex, and curved macroconidia (Fig. 3). Based on morphology and DNA sequence analyses, *F. semitectum* appears to be the fungal agent responsible for keratin degradation observed on *G. berlandieri.* Confocal microscopic examination confirmed the presence of the fungus within the interior of the scute (Fig. 4), as did the release of the fungus when the keratin was dissolved in KOH.

Caps of two experimental tubes epoxied onto tortoises were opened by the activity of the tortoises. The remaining seven experimental tubes were positive for fungal growth (Fig. 5). None of the nine control sites exhibited fungal growth. Microscopic examination of excised experimental scute exposed to the fungal mycelia confirmed that the fungus had penetrated the scute. No fungal hyphae were observed on the surface of the uninfected scutes nor in their sections. Neither of the 14 experimentals nor 14 control chambers glued onto the box turtles were positive for fungal growth.

Nine adult males and one female maintained at Laguna Vista had visible blemishes, as did all adults (9) and females (5) maintained at Donna. All of these tortoises were from the Lower Rio Grande River

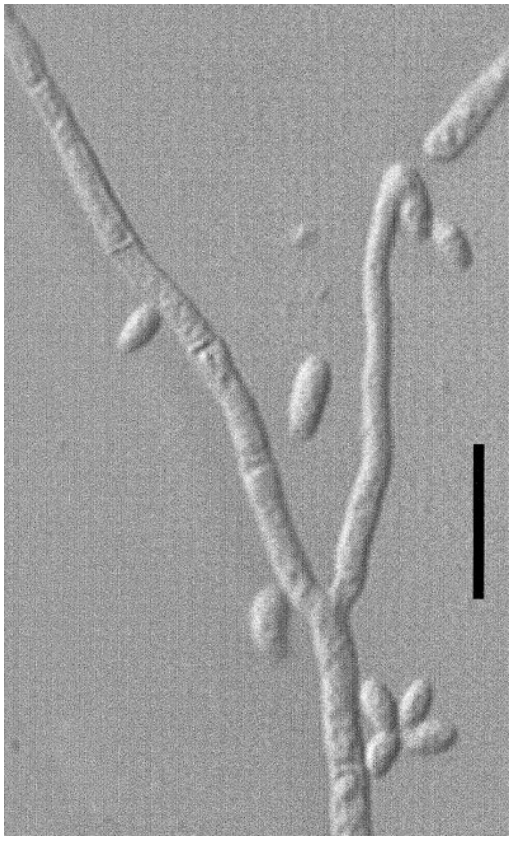

FIGURE 3. Isolated *Fusarium semitectum* hyphae showing septa, phialide with collarette at apex, and curved macroconidia. Bar = 20 μm.

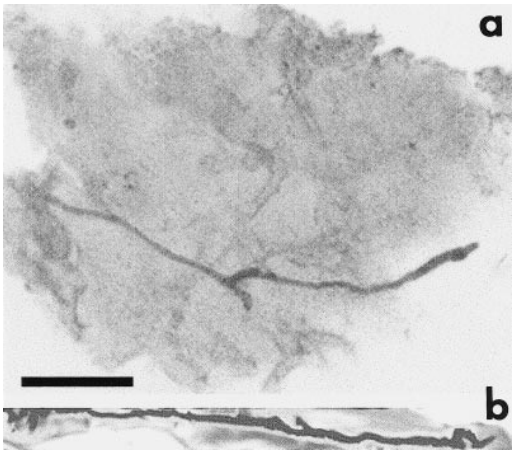

FIGURE 4. Scute disease in the Texas tortoise. (a) Confocal micrograph prepared by digital projection through multiple optical plains of infected tortoise scute fragment. (b) View corresponds to rotating the plan view 90°. The presence of a *Fusarium semitectum* hypha within the scute is clearly demonstrated. Bar = 50 μm.

Valley of Texas. In addition, all (15) juveniles hatched in 1977 and 1978 at Donna had extensive plastral blemishes. Eleven tortoise shells from Cameron and Hidalgo counties had extensive lesions attributed to the fungus. Only one shell of 27 tortoises from Dimmit and Zavala counties in western Texas exhibited a blemish, and that was <1cm in diameter.

DISCUSSION

Jacobson et al. (2000) and Jacobson (1980) reviewed mycotic diseases of reptiles but did not mention *F. semitectum* as a pathogen. Dyskerotosis (Jacobson et al., 1994), as observed in *G. agassizii,* may be similar to the condition reported here but the etiological agent(s) were not identified and bacterial associates were thought to be

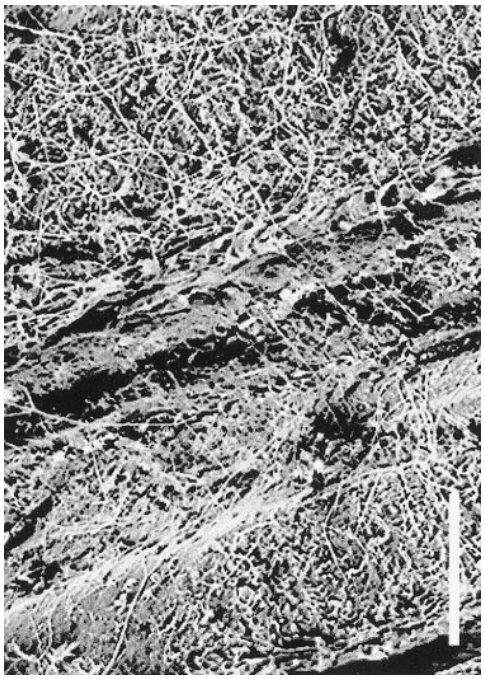

FIGURE 5. Scanning Electron micrograph of the scute surface of *Gopherus berlandieri* inoculated with a *Fusarium semitectum* suspension. Numerous hyphae are indicated. Control sites were free of hyphae. Bar = 100 μm.

secondary invaders. In addition, dyskerotosis induced high mortality. Many pathogens are opportunistic and readily invade exposed lesions making identification of the causative organism difficult. The inability of pathogens to readily degrade keratin or the metabolic products of *F. semitectum* degraded keratin in a dry environment probably limits participation.

The ability of the isolated *F. semitectum* to grow in keratin enhanced agar, where no other fungi grew, confocal microscopic confirmation that it penetrated the interior of the scute, and failure to observe the fungus in tortoises not exhibiting the whitish lesions eliminate the possibility that the fungus is a surface contaminant. This result was strengthened when it was confirmed that isolated fungal hyphae readily grew on, and penetrated, the scutes of *G. berlandieri.* Failure to observe the fungal lesions or to transfer the fungus to box turtles implies that there is a physical or chemical inhibition expressed by the scutes of these organisms.

It is not known whether the scute portion juxtaposed to bone is degraded and replaced or if it is not degraded. The fungus does not degrade new growth keratin at the edges of the scutes. It must obtain some sustenance other than keratin from the tortoise because scute degradation ceases after death of the host. In addition, the fungus is not keratin-dependent as it grew readily on a variety of carbon sources in culture.

It appears that the *F. semitectum* infections have a much higher prevalence in southern Texas than in the more inland areas of the tortoise's range. The keratin hooves of horses and cattle may be a reservoir and dispersing agent in the soil for this fungus, but it is more likely that the disease organism is widespread and that its expression in tortoises is limited geographically by local edaphic factors. Tortoises in the Lower Rio Grande Valley frequently construct shallow depressions called pallets (Auffenberg and Weaver, 1969) Use of these pallets is on a first-come-first-use basis, and on occasion the pallets are long enough to be shared by two tortoises simultaneously. Pallets appear to be less common in the soft sandy soils along the Dimmit-Zavala counties line, where tortoises frequently use burrows and holes dug by other animals. Tortoises using a pallet after it has been inoculated by fungal spores might be at higher risk of contacting the disease, especially if the tortoise remained in the pallet for an extended period, such as during the winter or through a drought.

Fortunately, the infections of *F. semitectum* on *G. berlandieri* progress slowly. There is no evidence that the disease is life threatening to the tortoises. As pointed out by Judd and Rose (2000), numerous people maintain high numbers of these tortoises in captivity. High artificial densities might enhance continued infections and the acquisition of new tortoises would possibly introduce differing strains of the fungus. When the tortoises can no longer be maintained in captivity, they are released or given to others to maintain, often with intra- and interspecific co-mingling. Although mycoplasma respiratory disease (Judd and Rose, 2000) is probably a more devastating ramification of these associations and releases, the threat of *F. semitectum* as a human corneal or nail pathogen should not be overlooked, especially in children.

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ment of Agriculture) who confirmed the species designation of the fungus based on PCR.

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