Identification of Earthworm Species on Golf Course Turf in Arkansas and Oklahoma

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Introduction

Relatively little research has been conducted on earthworm species composition in U.S. turfgrass systems, and what research has been done primarily focuses on non-native Asian and European species, if the species are listed at all (1-8). Preliminary observations at the University of Arkansas Agricultural Research and Extension Station suggested that the species present might belong to the North American-native *Diplocardia* genus. As morphological identification is difficult on the small *Diplocardia* spp. and nearly impossible on most juvenile earthworm species, DNA sequencing was used in this study to determine earthworm species composition of earthworms collected from golf course turfgrass systems in Arkansas and Oklahoma.

Objectives

The objective of this study was to determine earthworm species present on golf course turfgrass in the Arkansas-Oklahoma region.

Photo 1. A) Dig-and-sort collection and **B)** hand-sorting of earthworms.

Materials and Methods

- **Earthworm Collection**
  - Earthworms were collected from at least 3 subplots per sampling location (Table 1) by the dig-and-sort method (30 x 30 cm area to a depth of 20 cm, Photo 1A).
  - Earthworms were manually sorted (Photo 1B), counted (Table 2), boiled, and separated by general morphology (Photo 2).
  - Samples from Chenal Country Club, MeadowBrook Country Club, and the University of Arkansas were preserved in 95% ethanol at -80 °C. Samples from Jimmie Austin Golf Club and Lew Wentz Memorial Golf Course were preserved in 5% formalin at 24°C.
- **DNA Extraction**
  - DNA was extracted from 48 earthworms using a DNeasy® Blood & Tissue kit (Qiagen, Germantown, MD). Formalin-preserved samples were washed twice in phosphate buffer solution prior to lysing. Ethanol precipitation was used to further purify the extracted earthworm DNA.
- **DNA Amplification**
  - A 710-bp fragment of the mitochondrial cytochrome-c oxidase subunit gene was targeted for amplification using primers LCO1490 [GGT CAA CAA ATC ATA AAG ATA TTG G] and HCO2198 [TAA ACT TCA GGG TGA CCA AAA AAT CA] (9).
  - Amplification conditions were as follows: initial denaturation at 96°C for 10 m, 40 cycles of 95°C for 30 s, 50°C for 45 s, and 72°C for 1 m, and a final extension at 72°C for 5 m.
  - Amplification was confirmed by gel electrophoresis in 1.5% agarose gel and visualized by ethidium bromide fluorescence.
- **DNA Sequencing**
  - PCR products were purified using a Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI) and sent to Eurofins Genomics (Louisville, KY) for sequencing in both the forward and reverse directions.
- **Sequence Alignment and Adjustment**
  - Sequencher software (Gene Codes Corporation, Ann Arbor, MI) was used to trim, align, edit, and generate consensus sequences.
- **Tree Building**
  - Molecular Evolutionary Genetics Analysis ([MEGA7]; 10) was used to build a Neighbor-Joining tree (Fig. 1), which included 34 known sequences from the National Center for Biotechnology Information ([NCBI], Bethesda MD).

Table 1. Locations and earthworm counts from Arkansas and Oklahoma turfgrass systems sampled for earthworm identification.

Location	City	Latitude / Longitude	Turfgrass area sampled (number of samples)	Cultivar (if known)	Earthworm counts
Chenal Country Club	Little Rock, AR	34.778560 N 92.475937 W	Creeping bentgrass green (1) Zoysiagrass fairway (1) Zoysiagrass tee (1)	A-1 Meyer Cavalier	199
Jimmie Austin Golf Club	Norman, OK	35.188541 N 97.427982 W	Zoysiagrass tees (2) Bermudagrass fairway (1)	Zeon	19
Lew Wentz Memorial Golf Course	Ponca City, OK	36.730351 N 97.024931 W	Creeping bentgrass collar (3)		48
MeadowBrook Country Club	Tulsa, OK	36.042490 N 95.872778 W	Bermudagrass roughs (3)		41
University of Arkansas Agricultural Research and Extension Center	Fayetteville, AR	36.100231 N 94.168837 W	Bermudagrass tees (16)	Patriot	>600

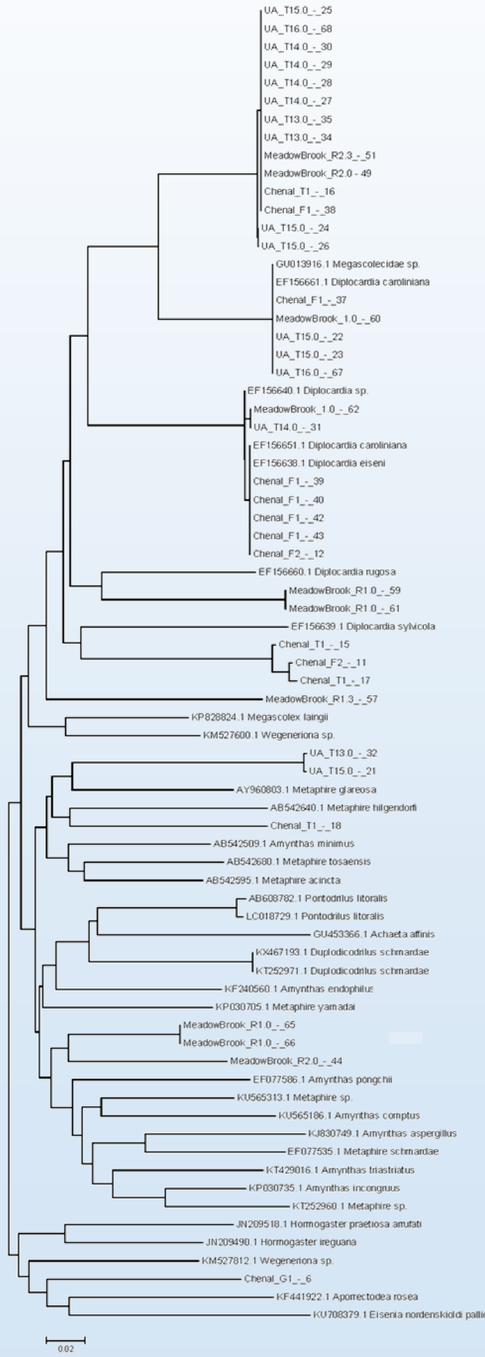

Figure 1. Neighbor-Joining Tree (unrooted) showing the relationship between earthworms collected from turfgrass systems in Arkansas and Oklahoma and known species from the NCBI database. Earthworms from this study are named by sampling location (Chenal Country Club, MeadowBrook Country Club, and the University of Arkansas Agricultural Research and Extension Station [UA]) and turfgrass area (F= Fairway, T=Tee box, and R= Rough).

Preliminary Results and Discussion

- 8 of the 48 earthworm samples analyzed did not yield DNA sequences.
  - 5 of these were formalin-fixed earthworm samples from Jimmie Austin Golf Club and Lew Wentz Memorial Golf Course.
  - Formalin cross-links DNA, resulting in amplification of primer-dimers.
  - 3 samples were from the Chenal tee, Chenal fairway, and a UA tee.
  - A sequence (from MeadowBrook) was removed due to poor alignment.
- 32 of the 48 earthworms (11 from Chenal, 7 from MeadowBrook, and 14 from UA) grouped with *Diplocardia* spp.
  - The *Diplocardia* genus is native to North America, and is thought to be more active in casting activity and over a wider range of temperatures than exotic earthworm species (11).
  - *Diplocardia* spp. were present in tees, fairways, and roughs.
  - Initial analyses indicate that *Diplocardia* spp. are prevalent, but that species richness is small.
  - *Diplocarida caroliniana* and *D. sylvicola* have previously been reported in Arkansas and Oklahoma (12, 13), though not near sampling locations.
  - *Diplocardia rugosa* has been reported in Oklahoma (13), though not near sampling locations.
  - *Diplocardia eiseni* has not been reported in either state.
- *Aporrectodea rosea* has been reported in Arkansas (12), though not near sampling locations.
- Six individuals (1 from Chenal, 3 from MeadowBrook, and 2 from UA) group with *Metaphire* and *Amynthas* spp.
  - *Metaphire* and *Amynthas* genera are from a primarily Oriental family (Megascolecidae) and were previously classified under the genus *Pheretima* (12).
  - One *Metaphire* spp. and three *Amynthas* spp. have previously been reported in Arkansas (12).
  - Four *Amynthas* spp. have previously ben reported in Oklahoma, but no *Metaphire* spp. have been reported (13).
- It has been suggested that *Metaphire, Amynthas,* and *Diplocardia* genera need revision and rearrangement (14).

Photo 2. A sample of earthworms collected Arkansas and Oklahoma turfgrass systems.

Ongoing Research

- Earthworm identification is being continued, to include morphological identification of adult specimens from Jimmie Austin Golf Club and Lew Wentz Memorial Golf Course.
- Analysis is continuing to confirm species relationships.
- Biomass and diversity will be measured.
- Correlation of species composition to earthworm casting activity at the University of Arkansas Research and Extension Center is being conducted.

Acknowledgements

Funding for this project was provided by the University of Arkansas Division of Agriculture and the United States Golf Association.

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