Using DNA Metabarcoding to Examine Wild Pig (*Sus scrofa*) Diets in a Subtropical Agro-Ecosystem

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ABSTRACT: The wild pig is well known for its generalist diet, a contributing factor to its successful invasion around the globe. We used DNA metabarcoding analyses of scat to examine wild pig diet on a cow-calf operation in south-central Florida. This 4,249-ha ranch is comprised of improved pastures and semi-native pastures that contain a mosaic of vegetation types. Both pasture types contain numerous wetlands and ditches as well as oak-palm woodlands. Fecal sampling was conducted along transects from March 2016 to February 2017. The study site was divided into five sampling areas to ensure dispersed sampling across the ranch. At least five freshly deposited scats were collected every two months from each sampling area and frozen. Regions of multiple genes that targeted either animal or plant DNA (CO1, trnL, and 12S rRNA marker genes) were selected for high throughput sequencing. Sequences were identified using the GenBank reference database. Two hundred nineteen fecal samples were collected and 196 were analyzed. Consensus lineages were retained if they could be confidently identified to family and were likely intentionally consumed by a pig. Between the three marker genes, 66 plant, 68 animal, and 12 fungal families were identified. Plant species dominated the diet with oak, torpedograss, joyweed, Bahiagrass, dayflower, and other grasses occurring in over half the samples analyzed. Animals were present across a wide taxonomic breadth, but encountered less frequently than plants with the exception of an exotic earthworm. Cattle, house mouse, cotton mouse, raccoon, mole cricket, Virginia opossum, and six species of fly were recorded from over 10% of fecal samples. This represents the first study to employ DNA metabarcoding to examine the dietary composition of this invasive vertebrate across an entire year.

KEY WORDS: agro-ecology, DNA metabarcoding, diet, invasive species, *Sus scrofa*, wild pig

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INTRODUCTION

The wild pig (*Sus scrofa*) is one of the most widely distributed mammals in the world and is classified as an invasive species outside its native range (Barrios-García and Ballari 2012). One factor that has allowed this species to establish itself in many regions is its broad and plastic omnivorous diet (Baubet et al. 2004, Irizar et al. 2004). Wild pigs also exhibit a wide range of feeding behaviors including browsing, grazing, foraging, rooting, and direct predation on animals (Loggins et al. 2002, Baubet et al. 2004). In both their native and introduced ranges, plants dominate their diet (Ballari and Barrios-García 2014). Both above-ground and below-ground vegetation is consumed – the latter made available through rooting.

Animals are frequently consumed but at a lower total volume compared to plants (Ballari and Barrios-García 2014). A wide variety of invertebrates and vertebrates have been documented as diet items (Henry and Conley 1972, Wood and Roark 1980, Ditchkoff and Mayer 2009, Wilcox and Van Vuren 2009, Robeson et al. 2018). Wild pigs are also important scavengers and are known to exhibit coprophagy (DeVault et al. 2003, Selva et al. 2003, Copado et al. 2004). Fungi are also present in wild pig diets, but are generally consumed at low frequencies (Ballari and Barrios-García 2014).

Wild pigs are able to adjust their diets based on food availability, that may vary regionally, seasonally, and across habitat types (Rollins and Carroll 2001, Wilcox and Van Vuren 2009). For example, diet items consumed while rooting are more frequently consumed when above-ground resources are low (Barrett 1978, Baron 1982). Herbaceous vegetation is often consumed during the early growing season (Wood and Roark 1980, Taylor and Hellgren 1997). Mast, particularly acorns, is consumed frequently during high mast years and dominates the diet in the fall and winter (Wood and Roark 1980, Loggins et al. 2002).

Wildlife diets are typically analyzed by macroscopic and microscopic visual examination of gut or fecal material (Schley and Roper 2003, Ballari and Barrios-García 2014). Due to digestive processes, confident identification can be limited to only recently ingested or

difficult to digest food items. Eggs and soft tissue, for example, may be impossible to identify visually even immediately after consumption. This has led to biased assessments of diet. In omnivorous species, such as the wild pig, estimation of dietary composition remains challenging. High-throughput sequencing, in particular DNA metabarcoding, allows for a more accurate assessment of diet as it does not rely on visual examination of gut or fecal contents (De Barba et al. 2014). DNA metabarcoding techniques have been successfully employed to examine diets in several ungulate species including wild pigs (Ait Baamrane et al. 2012, De Barba et al. 2014, Bergmann et al. 2015, Kartzinel et al. 2015, Robeson et al. 2018).

Although many past studies have examined wild pig diets across their native and introduced ranges, most have relied on gut content or fecal analyses (reviewed in Ballari and Barrios-García 2014) Conversely, Robeson et al. (2018) used DNA metabarcoding analyses to examine wild pig diet, but samples were only collected over a several week period. Our objective was to inventory the diversity of wild pig diet items on a Florida rangeland over 12 months. As such, our study is the first to examine wild pig dietary composition using DNA metabarcoding techniques over the course of an entire year.

METHODS

Study Area and Sample Collection

This study was conducted at Buck Island Ranch in southeast Highlands County, Florida. Buck Island Ranch is a 4,249-ha cow-calf operation with over 3,000 head of cattle. It is the location of the MacArthur Agro-Ecology Research Center (MAERC; Swain et al. 2013), which is a division of Archbold Biological Station. The ranch is comprised of improved pastures dominated by Bahiagrass (*Pasaplum notatum*) and semi-native pastures that contain a mosaic of Bahiagrass and native vegetation. Across the landscape are over 600 seasonal wetlands as well as hundreds of miles of ditches used for both drainage and irrigation. Within the semi-native pastures, oak-palm hammocks are also present. Buck Island Ranch supports diverse wildlife and plant communities including imperiled species such as wood storks (*Mycteria americana*), snail kites (*Rostrhamus sociabilis*), crested caracara (*Caracara cheriway*), and Edison's St. John's wort (*Hypericum edisonianum*).

Sample collection occurred between March 2016 and February 2017. We divided the year into six two-month periods and divided Buck Island Ranch into five roughly equivalent sampling areas to ensure dispersed sampling across the ranch. We collected at least five fecal samples from each sampling area during the following periods: March-April 2016, May-June 2016, July-August 2016, September-October 2016, November-December 2016, and January-February 2017. Within each sampling area we used the criterion that at least two samples had to be at least 500 m from the nearest sample. Fecal samples were collected on transects when possible along which we removed old scat the first day and collected freshly-deposited scat on subsequent days. If sufficient samples could not be collected by this method during a sampling period, we opportunistically searched for scat and collected presumably fresh samples that had a "greasy" appearance. Samples were collected and stored in plastic bags. Prior to freezing, we homogenized samples and aliquoted them into four separate bags. Samples were then stored at -20°C until analysis.

Laboratory Analyses and Data Curation

Three previously published PCR primer sets were used to amplify the corresponding targets listed here: a portion of the chloroplast trnL (UAA) intron – g (5'-GGG CAATCCTGAGCCAA-3') and h (5'-CCATTGAGTCT CTGCACCTATC-3') – to target plant taxa (Taberlet et al. 2007); the mitochondrial-encoded cytochrome oxidase subunit I (CO1) – (5'-GGWACWGGWTGAACWGTW TAYCCYCC-3') and (5'-CCNCCTCCNGCWGGRTCR AAARAA-3') – to target metazoan taxa (Carr et al. 2011, Leray et al. 2013); and the mitochondrially-encoded 12S rRNA subunit – (5'-ACTGGGATTAGATACCCCACT ATG-3') and (5'-GAGAGTGACGGGCGGTGT-3') – to target vertebrate taxa (Evans et al. 2016). DNA isolation and quantification as well as sequence processing were performed similarly as for trnL in Robeson et al. (2018) with the exception of using an updated version of the UNOISE (v3) pipeline. This pipeline generates representative OTU (Operational Taxonomic Unit) sequences in the form of Exact Sequence Variants (ESVs, Callahan et al. 2017) All laboratory analyses were conducted at Jonah Ventures, LLC (Boulder, CO).

Sequence records in FASTA format containing the trnL sequence fragment for plants, the CO1 sequence fragment for animals and fungi, and the 12S sequence fragment for vertebrates were downloaded from GenBank using Entrez Direct command-line tools (Benson 2004). We followed the methodology of Robeson et al. (2018) to extract the full taxonomic lineage for each record, and using NCBI BLAST, we selected the top hit that had an alignment query coverage and identity of at least 90% and 85% respectively. From these top hits we created an initial table of highest matching OTUs for each target marker for each sample.

Each OTU had a number of reads associated with each fecal sample. Prior to reviewing taxonomy for each OTU, we verified that samples were suid in origin. We compared the number of pig reads to the number of reads from other vertebrate species; if another vertebrate species had ten or more times as many reads than there were pig reads in a sample, that sample was considered non-suid in origin. We then removed all non-suid samples and their associated OTUs, all pig OTUs, and all human OTUs from the dataset.

Each remaining OTU defined in the previous step was reviewed with the corresponding FASTA sequence and re-input to NCBI BLAST to compare the resulting NCBI species lists to actual species occurrence data from the site, county, region, and state. We discarded any DNA sequences that could not be identified to family. Similarly, we discarded any sequences where the family level of the consensus lineage did not occur on-site. If we identified a consensus lineage to species, and only one member of its genus occurred in the area, we considered the consensus

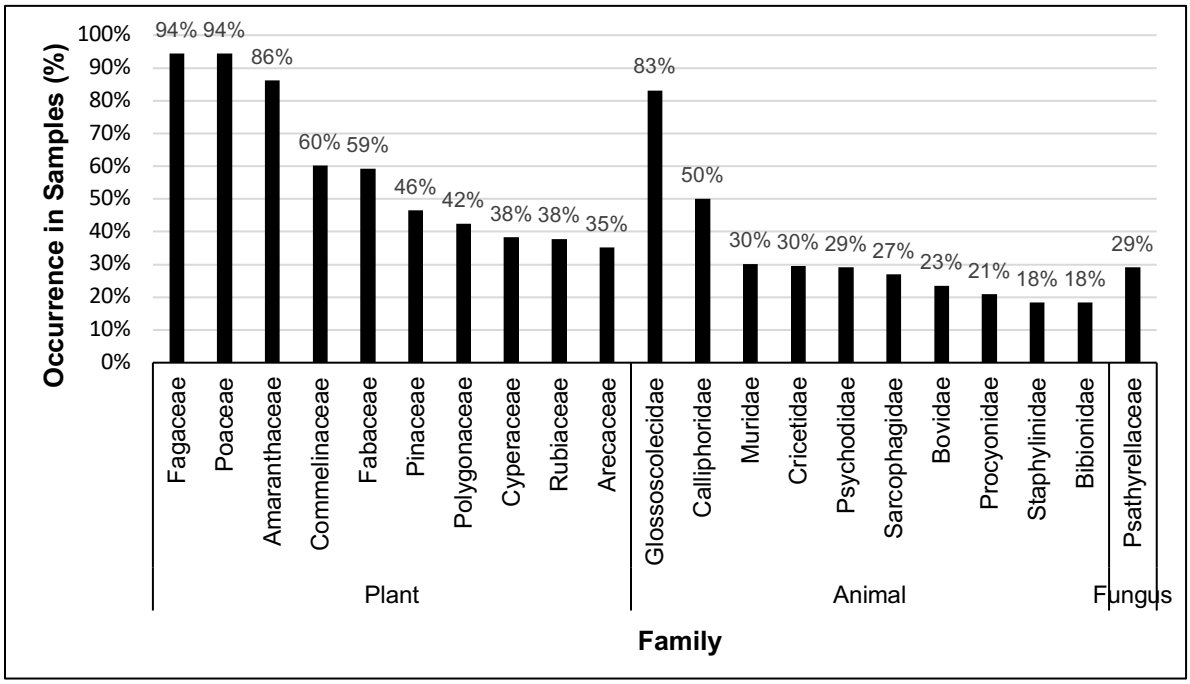

Figure 1. The top 10 most frequently occurring families for trnL (plants) and CO1 (animals and fungi).

lineage correct and the taxon was retained. If multiple species from a genus occurred on-site, the one with the highest BLAST score was retained, but only if the target marker for all local species was cataloged in GenBank. Conversely, if all species within a genus occurred on-site but were not represented in GenBank, only the genus was retained. We used the same rationale for assigning a consensus lineage to genus or family.

Plant (trnL) and vertebrate (12S & CO1) OTU data were verified with expert opinion and confirmed via the USDA PLANTS database (www.plants.usda.gov) and with site-specific species lists (www.maerc.org/html/data /specieslist.html). Arthropod and non-arthropod records (CO1) were verified using the Symbiota Collections of Arthropods Network (www.scan-bugs.org) and confirmed through expert opinion and primary literature (Reynolds 1994, Thompson 2000, Pierre et al. 2017). Fungi records (CO1) were confirmed by a mycologist (M. Smith, pers. comm.). In addition, the following higher taxa were immediately excluded from the CO1 data because they were determined to have either been unintentionally consumed by a pig or to have colonized the fecal samples prior to collection based upon their biology: fungi not belonging to Basidiomycota or Pezizales, rotifers (Phylum Rotifera), nematodes (Phylum Nematoda), sponges (Phylum Porifera), cnidarians (Phylum Cnidaria), worms belonging to the family Enchytraeidae, mites (Subclass Acari), water fleas (Order Cladocera), algae, gastrotrichs (Phylum Gastrotricha), and amoebas.

RESULTS

We analyzed 219 fecal samples. Of those, we discarded 23 samples. Six samples contained highly-degraded DNA and could not be used for analysis. The remaining 17 were non-suid in origin. This included samples from five cattle, four raccoons, three deer, two opossums, one coyote, one alligator, and one human. The results below are from the confirmed 196 good quality suid fecal samples.

trnL (UAA) gene

Eighty plant genera from 66 families were retained. Within those genera, 45 taxa were identified to species. The ten most commonly detected families in descending order were Fagaceae, Poaceae, Amaranthaceae, Commelinaceae, Fabaceae, Pinaceae, Polygonaceae, Cyperaceae, Rubiaceae, and Arecaceae (Figure 1). Oak (*Quercus* sp. [Fagaceae]), torpedograss (*Panicum repens* [Poaceae]), joyweed (*Alternanthera* sp. [Amarantheraceae]), Bahiagrass (*Paspalum notatum* [Poaceae], dayflower (*Commelina erecta* [Commelinaceae]), southern watergrass (*Luziola fluitans* [Poaceae]), and other grasses (Poaceae) occurred in over 60% of the fecal samples analyzed. An additional 11 taxa belonging to the families Pinaceae, Arecaceae, Polygonaceae, Fabaceae, Araliaceae, Asteraceae, Rubiaceae, Ceratophyllaceae, and Onagraceae were detected in over 25% of the fecal samples.

CO1 gene

Seventy-eight animal genera from 63 families were retained. Within those genera, 56 taxa were identified to species. The ten most commonly detected families in descending order were Glossoscolecidae, Calliphoridae, Muridae, Cricetidae, Psychopodidae, Sarcophagidae, Bovidae, Procyonidae, Bibionidae, and Staphylinidae (Figure 1). Animals were present across a wide taxonomic breadth, but generally encountered less frequently than plants

Table 1. A comparison of vertebrate taxa between CO1 and 12S.

Scientific Name	Common Name	CO1	12S
Fish			
Amia calva	Bowfin	1	2
Etheostoma fusiforme	Swamp Darter	1	0
Erimyzon sucetta	Lake Chubsucker	0	16
Notropis sp.	Eastern Shiner	0	1
Clarias batrachus	Walking Catfish	0	1
Amphibian			
Pseudobranchus axanthus	Southern Dwarf Siren	2	0
Siren sp.	Siren	4	0
Gastrophryne carolinensis	Eastern Narrowmouth Toad	2	0
Lithobates grylio	Pig Frog	0	4
Lithobates sphenocephalus	Southern Leopard Frog	0	8
Reptile			
Kinosternon subrubrum	Eastern Mud Turtle	2	0
Gopherus polyphemus	Gopher Tortoise	4	3
Alligator mississippiensis	American Alligator	1	2
Anolis carolinensis	Green Anole	1	0
Mammal			
Dasypus novemcinctus	Nine-Banded Armadillo	0	1
Didelphis virginiana	Virginia Opossum	14	6
Peromyscus gossypinus	Cotton Mouse	59	1
Mus musculus	House Mouse	48	63
Rattus rattus	Black Rat	14	0
Bos taurus	Cattle	48	73
Odocoileus virginianus	White-Tailed Deer	22	14
Procyon lotor	Raccoon	42	13
Canis latrans	Coyote	3	1

with the exception of an exotic earthworm [*Pontoscolex corethurus* (Glossoscolecidae)], which was detected in 84% of samples. Cattle [*Bos taurus* (Bovidae)], house mouse [*Mus musculus* (Muridae)], cotton mouse [*Peromyscus gossypinus* (Cricetidae)], raccoon [*Procyon lotor* (Procyonidae)], mole cricket (Gryllotalpidae), white-tailed deer [*Odocoileus virginianus* (Cervidae)], and six fly taxa (belonging to the families Calliphoridae, Bibionidae, Psychopodidae, and Sarcophagidae) were recorded from over 10% of samples.

Ten fungi genera from 12 families were retained. Within those genera, two taxa were identified to species, *Amanita rubescens* and *Pleuotus ostreatus*. *Psathyrella* sp. was detected in 28.8% of samples. No other taxa were detected in more than 10% of the samples.

12S rRNA gene

Fourteen vertebrate genera and 15 families were retained. Within those genera, 15 taxa were identified to species. The five most commonly detected families in descending order were Bovidae, Muridae, Catostomidae, Cervidae, and Procyonidae. Cattle was detected in 37.2% of samples and house mouse [was detected in 32.1%. Lake chubsucker [*Erimyzon sucetta* (Catostomidae)], white-tailed deer, and racoon were detected in greater than 5% of samples.

Using 12S primers we detected six taxa that were undetected during our CO1 analyses (Table 1). These included three fish [lake chubsucker, eastern shiner (*Notropis* sp.), and walking catfish (*Clarias batrachus*); two amphibians [pig frog (*Lithobates grylio*) and southern leopard frog [*L. sphenocephalus*)]; and one mammal [nine-banded armadillo (*Dasypus novemcintus*)]. Conversely, using CO1 primers we detected seven taxa that were undetected during our 12S analyses. These included one fish [swamp darter (*Etheostoma fusiforme*)]; three amphibians [southern dwarf siren (*Pseudobranchus axanthus)*], siren (*Siren* sp.), and eastern narrowmouth toad (*Gastrophryne carolinensis*)]; two reptiles (eastern mud turtle (*Kinosternon subrubrum*) and green anole (*Anolis carolinensis*); and one mammal [black rat (*Rattus rattus*)].

DISCUSSION

Multiple factors influence the diet of wild pigs, including food availability, energy requirements, seasonal variations, and regional variations (Ballari and Barrios-García 2014). Our data suggest wild pigs are consuming a wide variety of taxa at this site with 66 plant, 68 animal (CO1 and 12S combined), and 12 fungi families detected in their feces.

In general, plant taxa occurred more frequently than animal taxa. Oaks (*Quercus* sp.) are found in oak-palm hammocks throughout the site and 2016 was a high mast year. Elsewhere in their range, acorns have been shown to be a highly preferred diet item (Everitt and Alaniz 1980, Wood and Roark 1980), and their high occurrence in our samples was expected. Aside from two forbs, the most commonly occurring species were grasses. Notably absent from plant taxa detected was Carolina redroot (*Lachnanthes caroliniana*), which was targeted by wild pigs during a previous study at this site (Boughton and Boughton 2014) and detected by Robeson et al. (2018) in their Florida samples. Wild pigs tend to root when above-ground resources are scarce (Barrett 1978, Baron 1982), and because of the high acorn mast, they may not have heavily targeted Carolina redroot and other plants that require rooting during our sampling period.

Although most animals were generally detected less frequently than plants, a wide breadth of taxa were documented. Of particular interest was the exotic earthworm (*Pontoscolex corethurus*) because it was detected in the majority of samples. *Pontoscolex corethurus* is an exotic species from northern South America that has a circum-tropical distribution in croplands and other human-altered sites (Lavelle et al. 1987). Worms constitute an important part of wild pig diet elsewhere (Baubet et al. 2003). An additional invertebrate of interest is the mole cricket (Gryllotalpidae) due to its relatively high occurrence in samples compared to other taxa. Although we could not positively identify this record to genus, three common

species in Florida are invasive and can be serious pests of turfgrass and pastures including Bahiagrass (Kerr et al. 2017). These invertebrate species may be the target items which drive feral swine to root pastures and in turn cause loss to the livestock industry through subsequent forage degradation (Bankovich et al. 2016).

Vertebrates were well-represented in samples. The house mouse and cotton mouse were the most frequently observed vertebrates. Wild pigs are well-documented preying upon rodents at other sites (Ditchkoff and Mayer 2009, Wilcox and Van Vuren 2009, Robeson et al. 2018). Less clear are the high occurrences of cattle, white-tailed deer, and raccoon. Although direct predation upon juvenile deer and livestock has been recorded (Ditchkoff and Mayer 2009), this molecular approach cannot differentiate between predation, scavenging, or coprophagy (Robeson et al. 2018).

Similarly, we cannot positively explain the origin of many of the fly and beetle taxa occurring in our samples. The fly families Calliphoridae, Psychopodidae, Sarcophagidae, Bibionidae and the beetle family Staphylinidae account for five of the ten most commonly occurring animal families. All taxa are documented occurring on feces or carrion. Because we collected feces that had already been deposited in the environment, we cannot discern if these species colonized the feces prior to collection or, alternatively, were living on carrion or feces consumed by the pig.

Differences between vertebrate species recorded between CO1 and 12S markers are likely a result of both the lack of a related marker sequence from which to assign taxonomy within online databases and potential differences in amplification bias between the marker genes. For example, with the 12S marker we detected 16 occurrences of lake chubsucker, but zero occurrences with CO1. Similarly, although the trnL marker is very accurate at identifying plants to the family level, it has been recorded as only 67.3% accurate at unambiguously identifying a taxon to the species level (Taberlet et al. 2007). Oaks (*Quercus* sp.) were the most commonly detected diet item; however, this marker was unable to identify them to species.

Notably absent from both CO1 and 12S results were any bird species. Ground-nesting birds, in particular, have been documented in wild pig diets, which is a conservation concern for some species in Florida (Giménez-Anaya et al. 2008, Robeson et al. 2018). Our results also suggest limited nest predation of egg-laying reptiles at this site. Out of four gopher tortoise (*Gopherus polyphemus*) occurrences, one was recorded during the nesting season. Similarly, the only American alligator (*Alligator mississippiensis*) occurrence was in March, also outside the nesting season.

DNA metabarcoding for wildlife dietary analyses is not without its drawbacks as evidenced by the differences we saw between identification of vertebrate taxa from fecal samples in our CO1 and 12S results. Similarly, for some genera (e.g., *Quercus*) certain markers were unable to distinguish among species. Additionally, unless a sequence is already stored in a database (e.g., GenBank, BOLD) the correct taxon cannot be returned. This approach also cannot differentiate among the sources of diet items due to behavior. That is, it is generally not possible, unless directly observed, to discern the route by which the wild pig consumed the detected diet material (e.g., exhibited behaviors of scavenging, coprophagy, predation, and/or cannibalism). Finally, for this study in particular, using fecal samples collected from the environment rather than directly from the pig likely introduced DNA from taxa not consumed by pigs, particularly fly and beetle taxa.

Overall, we have corroborated that DNA metabarcoding is an efficient and effective method to analyze diet of an omnivorous wildlife species. This method allowed us to assess dietary composition from 196 fecal samples in weeks rather than the months it would have taken someone using macroscopic and microscopic visual examination. It also represents the first instance of cataloging the diversity of this invasive vertebrate's diet across an entire year. It should be noted that this molecular approach is not meant to fully replace other techniques. The technique comes with pitfalls, including comprehensiveness of local species catalogued in reference databases, the resolution at which particular metabarcoding sequences can be identified to lower taxonomic levels, and the requisite knowledge of local species composition and abundance. The utility and necessity of using traditional gut content analyses or behavioral studies to assess diet remain. Ultimately, the questions at hand should dictate the study design and methodology for any future wild pig diet studies.

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