EVALUATING THE RISK OF VERTEBRATE PATHOGEN TRANSMISSION VIA RIPARIAN RESTORATION

by

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ABSTRACT

Riparian restoration frequently involves planting native species sourced from plant nurseries or other riparian areas. This presents an opportunity for the introduction of novel plant pathogens; however, it is unknown if these practices could transmit pathogens affecting other taxa, such as aquatic and semi-aquatic vertebrates. I investigated the occurrence of *Batrachochytrium dendrobatidis* and ranavirus in plant nurseries and the Gila and Mimbres rivers in southwestern New Mexico using eDNA methods. Positive detections of *Bd* were sequenced to examine isolate diversity. *Bd* was only detected in one plant nursery wetbed actively used by *Spea* tadpoles. Ranavirus was not detected in plant nurseries. Both *Bd* and ranavirus were detected in the field. *Bd* isolates found in the plant nursery differed genetically from those found in either the Mimbres or Gila rivers. Thus, managers should be aware of the potential to introduce novel varieties to local host populations. My findings suggest that the risk of transmission is minimal and likely non-existent depending on the restoration methods employed. Finally, I make suggestions to effectively mitigate the risk of transmission if concerns are raised for restoration projects in sensitive habitats.

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This thesis is dedicated to Iris Elizabeth Todd, whose imagination and curiosity will light the world for decades to come.

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INTRODUCTION

Riparian zones make up a small percentage of the landscape in the semi-arid West of North America yet they provide benefits for both aquatic and terrestrial organisms (Patten 1998). Ecological functions of riparian zones include maintaining stream bank stability, stabilizing water temperatures, suppressing algal blooms in eutrophic waters, and facilitating the transfer of energy from the terrestrial system into aquatic food webs and vice versa (Barling and Moore 1994, Hancock et al. 1996, Hood and Naiman 2000, Richardson et al. 2007). Riparian zones also provide wildlife corridors, and in semi-arid western North America, are often among the few suitable habitats for supporting amphibians and invertebrates that require moist conditions (Naiman and Décamps 1997, Patten 1998). As a result, riparian areas play vital roles in creating and protecting regional biodiversity, particularly in arid ecosystems.

Riparian zones are heavily exploited and altered by anthropogenic activities such as dam construction, channelization, water extraction, logging, and grazing (Hancock et al. 1996, Patten 1998, Richardson et al. 2007, Nilsson and Berggren 2000). Because of the severity of damage to many riparian areas and their importance to ecosystem stability, they have become a priority for conservation efforts (National Research Council 2002).

Conservation of riparian ecosystems often involves restoration, which includes the removal of non-native flora and the planting of native woody and herbaceous species (Richardson et al. 2007). However, plants used for restoration projects are frequently sourced from outside the watershed being restored. This practice poses a risk of introducing plant pests and pathogens (Liebhold et al. 2012). However, we do not know if transmission of pathogens affecting vertebrate taxa is occurring through floral transplants. The present study examined the hypothesis that plant materials used in riparian restoration projects act as vectors in the transmission of pathogens that infect poikilothermic aquatic vertebrates. I focus on the pathogens *Batrachochytrium dendrobatidis* (hereafter *Bd*) and ranavirus which have caused considerable loss of biodiversity and in case of *Bd*, host extinctions (Scheele et al. 2019).

Chytridiomycosis is an infectious disease caused by the fungus *Batrachochytrium dendrobatidis* (Mendelson et al. 2006, Lips 2016). *Bd* is genetically diverse, representing numerous lineages of varying pathogenicity (Berger et al. 2005, Morgan et al. 2007, Retallick and Miera 2007). Thus, even if *Bd* already occurs locally, managers must avoid introducing a novel strain of the pathogen that could be potentially more lethal.

Ranaviruses infect aquatic poikilothermic vertebrates (e.g. amphibians, fish, and reptiles; Hoverman et al. 2011, Brunner et al. 2015, Duffus et al. 2015, Gray and Chinchar 2015). Symptoms of ranavirus infection include emaciation, lethargy, and ulceration of the skin which can lead to bleeding and ultimately death (Gray and Chinchar 2015). Ranaviruses have been linked to mass die offs of amphibians globally (Daszak et al. 2003). While direct transmission of ranavirus from host to host is most common, there have been documented cases where ranavirus has infected a host through

indirect transmission via contaminated water, soil, and sediment (Gray et al. 2009, Nazir et al. 2012, Brunner and Yarber 2018). Thus ranavirus could be introduced to new virus-free locations through the importation of foreign plants, or other organic materials.

The implications of introducing *Bd* and ranavirus into sensitive habitats such as the Mimbres and Gila watersheds are profound. Our most powerful management tool for conserving and restoring riparian habitats could inadvertently damage these communities. However, for pathogen transfer to occur, plant nursery facilities would need to act as reservoirs of *Bd* and ranavirus. To my knowledge, the occurrence of these pathogens in plant nurseries has not been documented. Likewise, knowledge of the genetic diversity of the pathogens among field and nursery samples (if present) is also absent. I examined the prevalence of *Bd* and ranavirus in the Mimbres and Gila Rivers and in 4 plant nurseries. I also determined if *Bd* from different locations represent distinct isolates of the pathogen. Finally, I explore possible risk mitigation strategies for future restoration projects to minimize the possibility of the introduction of these pathogens.

STUDY AREA

This study focused on 2 rivers, the Mimbres and Gila Rivers of southwestern New Mexico (Figure 1). The Mimbres River flows approximately 91 miles from its headwaters in the Gila National Forest within the Aldo Leopold Wilderness Area and terminating around Deming, New Mexico. The Mimbres River basin is approximately 4,600 square miles and is a closed basin. The Gila River's headwaters are located in the Gila National Forest. The Gila River basin is approximately 60,000 square miles, with the river flowing from New Mexico, through Arizona, before discharging into the Gulf of

California. Both rivers serve as critical sources of water for anthropogenic activities in the semi-arid environment of southwestern New Mexico. Both rivers also serve as habitat for several federally and state listed endemic species, many of which would be susceptible to *Bd* or ranavirus (e.g. Chiricahua Leopard Frog [*Rana chiricahuensis*], loach minnow [*Rhinichthys cobitis*], Gila topminnow [*Poeciliopsis occidentalis*], Gila chub [*Gila intermedia*], Chihuahua chub [*Gila nigrescens*], beautiful shiner [*Cyprinella Formosa*], spikedace [*Meda fulgida*], Gila trout [*Oncorhynchus gilae*], New Mexico Department of Game and Fish 2016). This high endemism involving threatened species makes the Gila and Mimbres Rivers prime candidates for riparian restoration projects by state and federal agencies as well as non-governmental organizations.

METHODS

A total of 34 water samples were collected from 28 locations on the Gila and Mimbres Rivers during 3 sampling seasons (spring 2019, fall 2019, and spring 2020). During spring (April) 2019 I sampled 3 locations on the Gila and 3 on the Mimbres rivers. During fall (October) 2019 I revisited the previous 6 locations and 8 additional sites. Finally, during spring (May) 2020 I sampled an additional 14 locations (figure 1). One-liter of river water was collected at each location, treated with Longmire's solution (Williams et al. 2016) and stored on dry ice for transport. Samples were taken at points of the river that were accessible by foot from the road or trails. The edge of the two rivers were the primary sample location as this serves as the primary habitat for amphibians. Samples were taken to the Palo Duro Research Facility, West Texas A&M University, and stored at -80 ºC.

Four plant nurseries were visited in spring and summer of 2019. All of the nurseries sampled were commercial facilities and had been involved in restoration projects in the past 10 years. The names of the facilities are withheld as part of their agreement to participate in this study. At each nursery, I collected five 1 L water samples and five 25 g soil samples (an additional soil sample was included for 2 nurseries). Samples were taken from a variety of sources based on the practices at the facility. For example, water samples were taken from water faucets, watering jugs, and wetbeds whereas soil samples were collected from pots, from soil bags, as well as previously used soil. Water samples were treated with Longmire's solution (Williams et al. 2016) and both soil and water samples were stored on dry ice and transported to the Palo Duro Research Facility, West Texas A&M University and stored at -80 ºC.

DNA extraction from water samples followed previously reported protocols (Kirshtein et al. 2007). Approximately 1 L of water from all sampling locations (rivers and nurseries) was filtered through 0.2 μm sterile filters. Filters were then rinsed by filtering sterile phosphate buffered saline (PBS). All filters were frozen at -80 ºC as soon as possible. DNA extractions were performed using the Qiagen AllPrep Fungal DNA/RNA/Protein kit (for *Bd*) or the Qiagen AllPrep PowerViral DNA/RNA kit (for Ranavirus). Except for the initial steps, the manufacturer's protocols were followed when isolating *Bd* and viral DNA from filters. During the initial steps, 0.9 ml of lysis buffer with 0.1 mg/ml proteinase K were added to the filter, the tube was sealed, and incubated at 55 °C for 1 h. Incubated tubes were rotated continually in order to bathe the filter in lysis mixture. Extracted DNA was stored at -80 °C. For soil samples the Qiagen DNEasy Power Soil kit (for *Bd*) and the DNEasy Blood and Tissue kit (for Ranavirus) was used

following the manufacturer's protocol for extracting DNA from soil. Extracted DNA was stored at -80 °C.

For PCR detection of *Bd* total eukaryotic DNA was first amplified using the EukA (5'-AACCTGGTTGATCCTGCCAGT-3') and 1195RE (5'-GGGCATCACAGACCTG-3') primer pair and ReadyMix Taq PCR Master Mix (Sigma) (Freeman et al. 2009a, b). Following amplification, amplified DNA was purified using a Qiagen PCR Cleanup kit. Purified DNA was then used as a template to amplify *Bd* specific DNA using a highly cited qPCR probe-based protocol amplifying the highly specific Internal Transcribed Spacer (ITS1) genomic region (Boyle et al. 2004). 25 µl reactions each containing 12.5 µl 2 × iTaq Universal Probes Supermix (Bio-Rad), PCR primers ITS1-3 Chytr (5'-CCTTGATATAATACAGTGTGCCATATGTC-3') and 5.8S Chytr (5'-AGCCAAGAGATCCGTTGTCAAA-3') at a concentration of 900 nM each. The Minor Groove Binding (MGB) probe (Chytr MGB2; 5'-6FAM CGAGTCGAACAAAAT MGBNFQ-3') at 250 nM and 5 µl of purified eukaryotic DNA was used to detect *Bd* using a BioRad CFX96 Real-Time qPCR detection system. Amplification conditions were set at 2 min at 50 °C, 10 min at 95 °C followed by 15 s at 95 °C and 1 min at 60 °C for 50 cycles. For each sample, qPCR tests for *Bd* were performed in triplicate.

qPCR samples that tested positive for the presence of *Bd* were subjected again to conventional PCR using the ITS1-3 and 5.8S Chytr primers following the conditions above as strains of *Bd* differ in number and identity of ITS1 haplotypes (Longo et al. 2013). PCR products were visualized on an ethidium bromide-infused agarose gel and then gel-purified using a Qiagen Gel Purification kit. PCR products were cloned into

JM109-competent cells following the manufacturer's instructions for the pGEM-T Easy Vector System I (Promega), as previously described (Hydeman et al. 2017). Blue/white screening was used to identify successfully transformed colonies. Five positive colonies per sample were incubated at 95 °C for 10 min in 25 µl of sterile water, and DNA was amplified one last time using the M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') primer pair. DNA sequencing was performed at the Molecular Core Research Facility at Idaho State University.

For the PCR detection of Ranavirus, samples were analyzed using a BioRad CFX96 Real-Time qPCR detection system using primers and probes that amplify a small region in the ranavirus Major Capsid Protein (MCP) (Warne et al. 2016). Reactions contained 5 µl extracted viral DNA, 12.5 µl 2 × iTaq Universal Probes Supermix (Bio-Rad), 300 nmol of forward rtMCP primer (5'-ACACCACCGCCCAAAAGTAC-3'), 900 nmol reverse rtMCP primer (5'-CCGTTCATGATGCGGATAATG-3'), and 250 nmol of rtMCP probe (5'-FAM CCTCATCGTTCTGGCCATCAACCAC TAMRA-3). Amplification conditions were set at 2 min at 50 °C, 10 min at 95 °C followed by 15 s at 95 °C and 1 min at 60 °C for 50 cycles. For each sample, qPCR tests for ranavirus were performed in triplicate.

RESULTS

Bd and ranavirus DNA were detected in water samples collected from the Gila and Mimbres rivers (Table 1). Of the six locations sampled during the spring (April) of 2019, *Bd* was detected in the Mimbres River near the town of Faywood, NM (3/3 qPCR replicates were positive; Figure 3) and on the Gila River at the Gila Campground (1/3

qPCR replicates were positive). Ranavirus was not detected during the spring 2019 sampling. Of the 14 locations sampled during the fall (October) 2019 field sampling, ranavirus was detected in the Gila River at the Hwy 211 bridge near Cliff, NM and at the bridge at Red Rock, NM (for both 1/3 qPCR replicates were positive). Ranavirus was also detected on the Mimbres River at McKnight Rd. *Bd* was not detected in samples collected in the fall of 2019. Finally, of the 14 locations sampled during the spring 2020 (May), we did not detect either pathogen.

Our overall detection rate for *Bd* at river sample sites was 5.9% (2/34 samples). This was lower than a previous study which tested individuals for *Bd* in southwestern New Mexico at a 14.6% detection rate for sampled locations (Christman and Jennings 2018). This difference in positive sample locations could be caused by the difference in methodology, the previously mentioned study tested individuals by swabbing the ventral surface of the body with a cotton swab and extracting the DNA from the cotton swab to run through PCR analysis. This difference could also have been caused by number of sample locations taken, and our sample locations being biased to areas with high anthropogenic activity. This could cause lower amphibian populations along these parts of the river which could lead to lower detection rates. To my knowledge, no other study has used eDNA to test for *Bd* in the environment in this area of New Mexico.

In addition, water samples taken from one of the 4 plant nurseries tested positive for *Bd* (Table 2). At Plant Nursery 1, samples positive for *Bd* were collected from wetbeds occupied by *Spea* tadpoles. Bd was not detected in soil from nurseries (Table 2).

Ranavirus was not detected in soil or water obtained from the sampled nurseries (Table 2).

For each positive *Bd* sample (n=34), 5 clones were sequenced. Sequence analyses using MacVector 17.5.5 revealed that each positive *Bd* sample consisted of the same isolate (e.g., all 5 clones were identical). The 3 *Bd* positive samples from plant nursery 1 were of the same isolate (GenBank Accession Number MW147112). The *Bd* positive water samples from the Gila River (Gila Campground; GenBank Accession Number MW147110) and the Mimbres River (near Faywood, NM; GenBank Accession Number MW147111) both collected in the spring of 2019 were unique from one another (4.03% variation). The positive sample from Plant Nursery 1 was also different from both the Gila and Mimbres samples (Plant Nursery 1 – Mimbres River samples were 5.33% different; Plant Nursery 1 and Gila River samples were 6.04% different). Overall variation between sequences was 5.41% (Ibid).

DISCUSSION

Riparian zones are vulnerable habitats that will be at the forefront of conservation efforts in the arid Southwest for the foreseeable future (Smith et al. 2014, Friberg et al. 2016). Restoration of these communities will require planting of native flora which is linked to a risk of introducing plant pathogens (Sims et al. 2019). I provide the first evaluation of the potential risk of transferring pathogens of poikilothermic aquatic vertebrates from plant nurseries into riparian habitats by evaluating pathogen occurrence and lineage diversity in plant nursery facilities (however, see Johnson and Speare 2005). My results yield two major findings: 1) Pathogens such as *Bd* can occur in plant

nurseries. Thus, the possibility of introducing these pathogens into riparian systems via transplanted flora is a real, albeit small possibility; 2) My results support previous assessments that pathogens, such as *Bd* are diverse. Thus, managers should consider not only the possibility of spreading pathogens but also introducing novel strains which may be more lethal than native varieties.

I detected *Bd* in a series of wetbeds at one plant nursery. These wetbeds were actively being used by *Spea* tadpoles. Structures actively being used by amphibians or fish are obviously more likely to contain *Bd* or ranavirus. Likewise, plant materials grown in structures used by hosts are more likely to transmit pathogens as part of a restoration project. However, this was my only detection of pathogens in plant nurseries. Amphibians are a common occurrence in plant nursery facilities. However, they typically are not associated with deposits of soil or potted plants in my observation. Typically, commercial facilities move plants and soil regularly (potting plants, moving inventory, etc.). This activity disturbs animals and entices them to find quieter habitats. In addition, the water sources from the sampled plant nurseries either came from municipal water sources or well water. Both are unlikely to be reservoirs of *Bd* or ranavirus because of chemical treatment in municipal water supplies inaccessibility to vertebrate hosts. Greenhouses often reach temperatures of above 30 ºC in the spring and summer months, which exceeds the optimal temperature range for *Bd* growth (Johnson et al 2003, Piotrowski et al. 2004). However, these temperatures may not inhibit the growth of ranavirus (Ariel et al. 2009, La Fauce et al. 2012). My findings do not address the likelihood that pathogens would survive transport into the field. However, previous work

suggests that *Bd* and ranaviruses are robust and capable of surviving in soil and water (Johnson and Speare 2003, 2005; Nazir et al. 2012).

There has been much discussion regarding which isolates of *Bd* are novel versus endemic and how they vary in pathogenicity. The picture that has emerged suggests that *Bd* isolates can vary greatly even at small geographic scales (Berger et al. 2007, Morgan et al. 2007, Retallick and Miera 2007). In addition, researchers have identified groups of isolates (i.e. lineages) that are highly virulent and spread due to anthropogenic activities (Rachowicz et al. 2005, Fisher et al. 2009, Farrer et al. 2011). Local adaptation of host populations and coexistence with *Bd* has been demonstrated (Palomar et al. 2016, Christie and Searle 2018). Introduction of novel isolates, for which host populations have limited immunity could be catastrophic (Schloegel et al. 2012). *Bd* positive samples collected from the Mimbres and Gila Rivers as well as the positive detections from the wetbeds represented different isolates. Thus, *Bd* is diverse among natural habitats as well as plant nursery facilities and it is not as simple as introducing pathogens into previously pathogen free habitats. This is an important consideration in sourcing plant materials. It should not be considered a safe practice to source plants from localities (e.g. other riparian habitats, plant nurseries) if both the source location and the restoration site have a history of pathogen occurrence. Movement of plant materials could introduce a new, potentially more lethal varieties of pathogens.

MANAGEMENT IMPLICATIONS

Previous work has provided strategies for reducing infection load in individuals (e.g. increasing body temperature to clear *Bd*) as well as prophylactically treating

individuals and habitats with chemicals to reduce the prevalence of the pathogens before species reintroduction programs (Johnson et al. 2003, Woodhams et al. 2011, Langwig et al. 2015, Bataille et al. 2016, Garner et al. 2016, Hudson et al. 2016, Geiger et al. 2017, Heard et al. 2018, Hettyey et al. 2019). While molecularly testing the restoration sites and potential plant sources can be a strategy taken to reduce the risk of introducing novel pathogens. This approach can be costly and take time, both of which may not be feasible for the restoration project. However, to my knowledge no other mitigation strategy has been developed for minimizing risk of introducing *Bd* and ranavirus during riparian restoration projects. Here I provide strategies for restoration managers to consider when planning projects: First, my results suggest that the risk of transmission is very small and may strongly depend on the planting methods employed. For example, planting from seeds or cuttings (e.g. cottonwood "sticks" commonly used in restoration projects) offers minimal risk due to the lack of soil and moisture which may harbor *Bd* or ranavirus. Second, I recommend that plant nurseries consider taking measures to prohibit the use of wetbeds by amphibians or fish (physical barriers or direct removal) and the application of disinfectants (see below) if they intend to use those plant materials in restoration projects. However, I see no reason for wildlife managers to avoid working with plant nurseries that employ wetbeds. I detected *Bd* in wetbeds at one nursery, but detected no pathogens elsewhere in the facility. Third, I found evidence of lineage diversity of *Bd* among sites. Thus, if plants are sourced from natural habitats I recommend collecting material from within the same drainage as restoration sites. A final option is to treat plant materials prophylactically, with effective disinfectants that do not harm them. For example, Virkon S (Lanxess Inc., active ingredients: 20.4% potassium peroxymonosulfate and 1.5%

sodium chloride) is a commercially produced broad-spectrum disinfectant which is well suited for disinfecting field equipment to prevent the transmission of *Bd* and ranavirus (Bryan et al. 2009, Gold et al. 2013, Rooij et al. 2017). Li et al. 2015 showed the effectiveness of Virkon S as a preventive treatment for pathogens in tomato production. The authors applied Virkon S at a concentration up to 2%. I expect plants such as cottonwoods and willow (Salix spp.) cuttings to tolerate Virkon S, particularly if a 1% concentration (as recommended for equipment decontamination) is applied. Restoration project managers should work with greenhouse operators and consult the literature for new chemical treatments if potential risks are identified.

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Table 1. *Bd* and ranavirus eDNA qPCR results for water samples collected in the Gila and Mimbres drainages. Samples were considered positive if one qPCR replicate of three was positive. The number of positive samples and the total number of samples are provided in parentheses.

	Pathogen	
Sample	*Bd*	Ranavirus
Spring 2019	33.3% (2/6)	0.0% (0/6)
Fall 2019	0.0% (0/14)	20.0% (3/14)
Spring 2020	0.0% (0/14)	0.0% (0/14)
Total	5.9% (2/34)	8.8% (3/34)

Table 2. *Bd* and ranavirus eDNA test results for water and soil samples collected at four plant nurseries previously involved in riparian restoration projects on the Gila and Mimbres rivers. Samples were considered positive if one qPCR replicate of three was positive. The number of positive samples and the total number of samples are provided in parentheses.

	Soil		Water	
Site	*Bd*	Ranavirus	*Bd*	Ranavirus
Plant Nursery 1	0.0% (0/5)	0.0% (0/5)	60.0% (3/5)	0.0% (0/5)
Plant Nursery 2	0.0% (0/6)	0.0% (0/6)	0.0% (0/5)	0.0% (0/5)
Plant Nursery 3	0.0% (0/6)	0.0% (0/6)	0.0% (0/5)	0.0% (0/5)
Plant Nursery 4	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)	0.0% (0/5)
Total	0.0% (0/22)	0.0% (0/22)	15.0% (3/20)	0.0% (0/20)

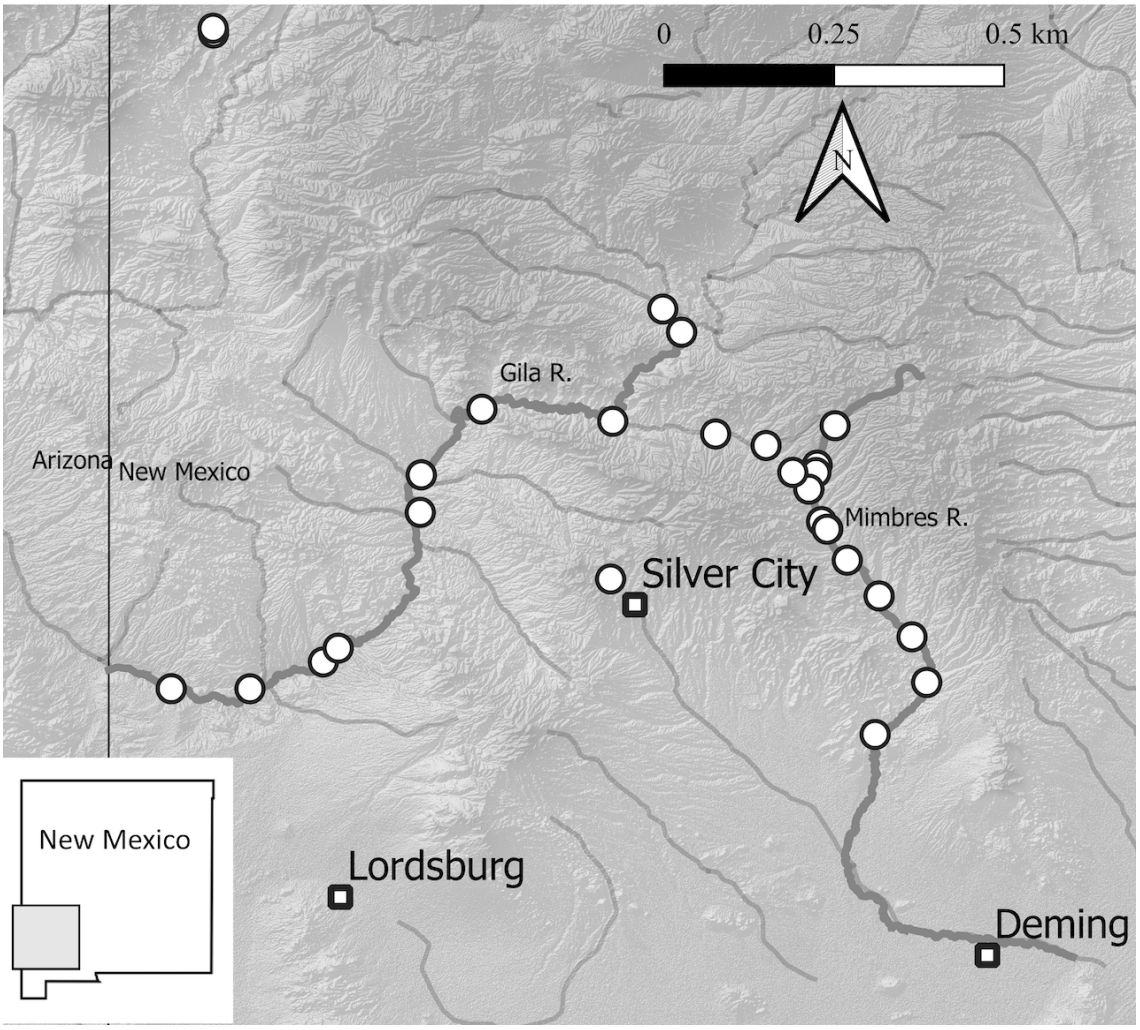

Figure 1. Map illustrating the eDNA water sample collection locations in southwestern New Mexico.