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Share with Wildlife FY2019

Evaluation of the risk of novel pathogen transmission via riparian restoration on the Mimbres River of southwestern New Mexico

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Abstract

Human activities have dramatically altered the rivers of the American Southwest over the last century, thus necessitating the restoration of riparian habitats. Yet, the process of restoration is not without risk. Riparian restoration often involves the reestablishment of native plant communities by planting. This often requires that plant materials be sourced from plant nursery facilities. Plant materials from plant nurseries may carry plant pathogens that could infect naturally established plants at the transplant site. However, it is unknown if plant materials could also transmit pathogens affecting aquatic vertebrates, such as *Batrachochytrium dendrobatidis* (hereafter *Bd*) and ranavirus, two pathogens that are well documented as causing die-offs and extinctions. *Bd* has profoundly affected amphibian communities globally as well as in the Western US, where it has played a role in the decline of the boreal toad (*Anaxyrus boreas*), Chiricahua leopard frog (*Rana chiricahuensis*), and low land leopard frog (*Rana yavapaiensis*). To date, no extinctions caused by ranavirus have been documented, but the virus has caused die-offs in North and South America, Europe, Africa, and Asia. Ranavirus has been documented in the Chiricahua leopard frog (*Rana chiricahuensis*) and presents a threat to sport fisheries due to its wide host generality, infecting both fish and amphibians. Our objectives were threefold. First, we sought to determine the prevalence of the focal pathogens (*Bd* and ranavirus) in the Mimbres and Gila rivers and in plant nurseries. Second, if present, we determined if *Bd* from different locations represent distinct isolates/strains. Third, given our findings, we suggest risk mitigation strategies for future restoration projects. We detected *Bd* in one plant nursery, and only in a wetbed that was actively used by anurans. Both *Bd* and ranavirus were detected in the Gila and Mimbres rivers (*Bd*: 2/34 samples, ranavirus: 3/34 samples). The *Bd* isolates detected in the plant nursery were not genetically identical to the positive detections found in either the Mimbres or

Gila rivers. This suggests that while uncommon, pathogen lineage diversity may vary geographically. We suggest that transmission of these pathogens via the use of nursery plants for restoration activities is unlikely but not impossible. We conclude with three points for managers to consider when establishing strategies to minimize the risk of pathogen introduction during riparian restoration projects. First, plants grown in wetbeds may pose a risk of transmitting these pathogens. Second, given the lineage diversity we observed for *Bd*, sourcing plants from within the drainage of the restoration site would minimize risk. However, this may not be feasible in many circumstances. Finally, we suggest a chemical disinfectant that could be used prophylactically on plant materials to minimize the risk of transmitting *Bd* and ranavirus but should have minimal effect on plants.

Introduction

Riparian zones are important transitional habitats between aquatic and terrestrial ecosystems that provide a diverse array of ecosystem services. They are affected by fluvial processes, such as flooding and alluvial soil deposits, which create habitat for diverse plant communities (Gregory *et al.* 1991). Riparian flora contribute to the stabilization of the stream banks, moderation of water temperature, delivery of food, filtering of sediments, and control of nutrients (Barling & Moore 1994, Hood & Naiman 2000, Richardson *et al.* 2007). Riparian zones can also be corridors of movement for fauna and offer suitable habitat for more permanent residents, such as amphibians (Naiman & Décamps 1997). As a result, riparian zones play a critical role in protecting biodiversity.

However, riparian ecosystem degradation, as a result of anthropogenic activities, is a widespread problem (Richardson *et al.* 2007). For example, erecting dams and regulating flow

rates can have an impact on river channel formation (Nilsson & Berggren 2000). Land use in riparian zones, such as grazing and associated trampling, recreation, logging, and water extraction, can affect sediment deposition and eutrophication in rivers and streams (Hancock *et al.* 1996, Patten 1998). In addition, introduction of invasive competitors and novel pathogens can be devastating for riparian communities (Bailey *et al*. 2001, Liebhold *et al*. 2012, Sims *et al.* 2019).

Due to their importance to community structure and biodiversity, the maintenance and restoration of riparian zones is a priority for many conservation programs (National Research Council 2002). Riparian-zone restoration projects often involve restoring native plant communities by planting native species of woody and herbaceous plants (Richardson *et al.* 2007). The plants intended for transplantation are frequently sourced from outside the location being restored, such as other drainages or plant nursery facilities, due to the sites in need of restoration often not having large stands of native plants present for plant material collection purposes. Using translocated flora does pose a risk of introducing exotic plant pathogens and non-native pests into previously pathogen free environments, such as restoration sites (Liebhold *et al*. 2012). However, we currently know little regarding the risk of translocated plant material leading to the introduction of aquatic vertebrate pathogens (suggested as a possibility by Johnson & Speare 2005). The focus of this study was to evaluate the possibility that plant materials used in riparian restoration projects may act as vectors in the transmission of pathogens of aquatic vertebrates. We focus on the pathogens *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus, which affect aquatic vertebrates and have caused considerable loss of biodiversity.

The fungus *Bd* is the pathogen that causes the infectious disease chytridiomycosis (hereafter chytrid; Lips 2016). Chytrid occurs when *Bd* zoospores encyst in an amphibian host's

keratinized tissues (e.g., larval mouthparts, adult epidermis) and develop into zoosporangia (Longcore *et al.* 1999, Letcher *et al.* 2006). The progression of chytrid results in lesions, thickening of skin, impaired respiration, and ultimately death (Kilpatrick *et al.* 2010). Chytrid affects at least 33 families and over 500 species and has been responsible for a total of 90 amphibian extinctions (Scheele *et al.* 2019). This has been described as the greatest documented loss of biodiversity linked to a pathogen (Scheele *et al.* 2019). Ranaviruses infect aquatic poikilothermic vertebrates (e.g., amphibians, fish, and aquatic reptiles; Hoverman *et al.* 2011, Brunner *et al.* 2015, Duffus *et al.* 2015, Gray & Chinchar 2015). Symptoms of ranavirus infection include lethargy, emaciation, and ulceration of the skin to the point of bleeding, which ultimately leads to death (Gray and Chinchar 2015). Ranaviruses have been found in over 175 species across all continents except for Antarctica (Gray and Chinchar 2015). Locally, the occurrence of these pathogens is highly variable (Ridenhour and Storfer 2008, Lips 2016, Miller *et al.* 2018). Adding to this complexity is the fact that any given pathogen may have a number of related lineages (strains or isolates) across their range that may vary in pathogenicity (Berger *et al.* 2005, Schock *et al.* 2009). Introduction of novel strains could have negative impacts on native populations. For example, introduction of species (e.g., bullfrogs [*Rana catesbeiana*]) that host these novel strains has been demonstrated as a potent driver of local mortality events and extinctions globally (Schloegel *et al.* 2009. Urbina *et al.* 2018).

Could soil and water from plant nurseries represent a transmission vector for these pathogens (suggested by Johnson and Speare 2005), especially novel lineages? Previous work suggests that both *Bd* and ranaviruses can persist in the environment (soil and water) depending on conditions (Johnson and Speare 2003, Johnson and Speare 2005, Nazir *et al.* 2012, Brunner *et al.* 2018). In addition, *Bd* and ranaviruses have been shown to be dispersed by anthropogenic

means (Schloegel *et al.* 2009). Thus, concern regarding the use of nursery grown plants for restoration projects, especially in areas with vulnerable amphibians or other susceptible species present, is justified and may represent an obstacle for approval of future riparian restoration projects by agencies with regulatory authority. Knowledge of pathogen prevalence in plant nurseries, and presence of novel strains different from those found in natural systems, could inform decision making and determine the need for mitigation strategies to minimize the risk of transmission associated with the use of nursery grown plants in restoration efforts. This would be valuable for all restoration projects but particularly for those in threatened and diverse systems, such as those found the arid Southwestern US and Northwestern Mexico.

This project assesses the risk of pathogen introduction, including that of novel strains of *Bd*, as a result of riparian restoration on the Mimbres River of southwestern New Mexico. Approval of riparian restoration projects on the Mimbres entailing use of plant materials outside the Mimbres River drainage has been limited by concern that those activities could introduce pathogens, including novel strains of already extant pathogens, into the system. Concern is warranted as the Mimbres and nearby river systems, such as the Gila, are habitat for several federally listed species and Species of Greatest Conservation Need that would be susceptible to the focal pathogens for this project (e.g., Chiricahua Leopard Frog [*Rana chiricahuensis*], Gila trout [*Oncorhynchus gilae*], loach minnow [*Rhinichthys cobitis*], Gila topminnow [*Poeciliopsis occidentalis*], Gila chub [*Gila intermedia*], Chihuahua chub [*Gila nigrescens*], beautiful shiner [*Cyprinella Formosa*], and spikedace [*Meda fulgida*]; New Mexico Department of Game and Fish 2016).

In the current project we ask the following:

1. What is the prevalence of the focal pathogens (*Bd* and ranavirus) in the Mimbres and Gila rivers and in plant nurseries often used as sources for restoration materials?
2. If present, does *Bd* from different locations represent distinct isolates/strains of the pathogen? And do the *Bd* strains found along the Gila and Mimbres rivers differ from any found in samples from plant nurseries?
3. Given our findings, can we suggest risk mitigation strategies for restoration projects in sensitive riparian communities such as the Mimbres drainage?

Methods

<u>Fieldwork</u>

Fieldwork was performed during the spring of 2019, fall of 2019, and spring of 2020. During the spring (April) of 2019, we sampled three locations on both the Gila and Mimbres rivers (six locations total). During the fall (October) of 2019, we revisited the previous six locations and included eight additional sites within the approved sampling area (14 locations total). Finally, during the spring (May) of 2020, we sampled an additional 14 sampling locations as part of an approved extension of the project. All sampling locations are shown in Figure 1. 1 L 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

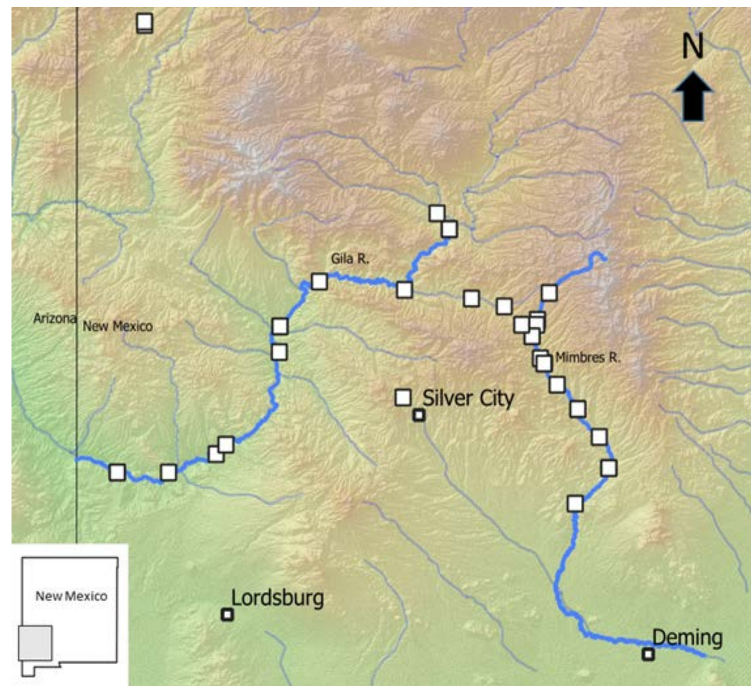

Figure 1. Map illustrating the eDNA water sample collection locations in southwestern New Mexico.

then returned to the Palo Duro Research Facility at West Texas A&M University and stored at -80 ºC.

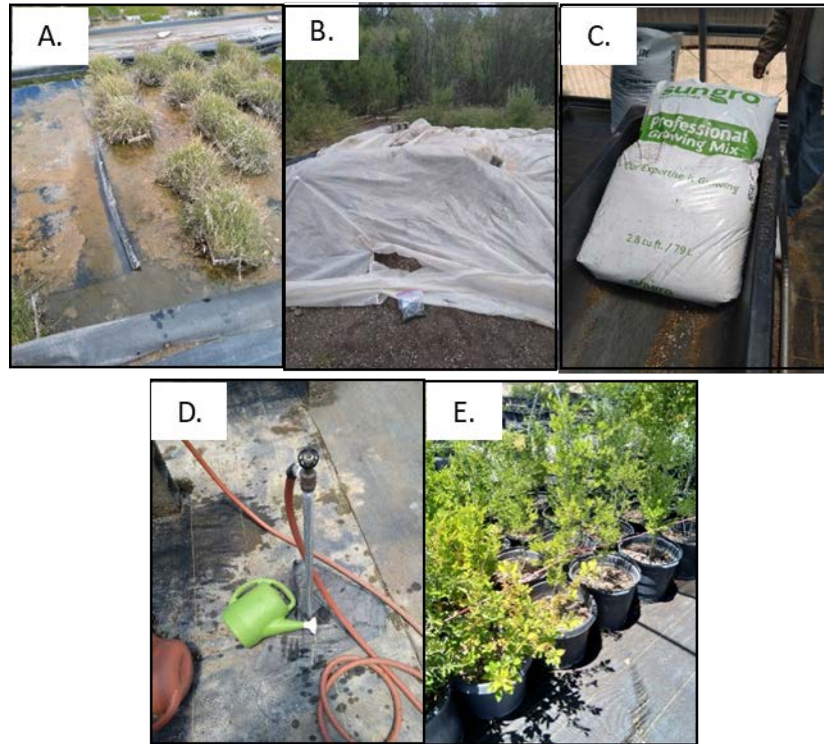

Figure 2. Selected examples of materials sampled at plant nurseries. A: wetbeds, B: locally sourced soil, C: commercial potting soil, D: water supplies, E: soil from plotted plants.

Four plant nurseries were visited in spring and summer of 2019. All of the nurseries sampled are commercial facilities and have been involved in restoration projects in the past 10 years. At each nursery, we collected five 1 L water samples and five 25 g soil samples. Samples were taken from a variety of sources based on the facility (Figure 2). For example, water samples were taken from water faucets, watering jugs, and wet beds. Soil samples were collected from pots, directly from soil bags, and from stores of 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 at West Texas A&M University and stored at -80 ºC.

Laboratory Methods

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*, n = 55 extractions) or the Qiagen AllPrep PowerViral DNA/RNA kit (for ranavirus, n = 55 extractions). Except for the initial steps, the manufacturer's protocols were followed for both when isolating fungal or 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 the tube containing the filter was incubated at 55 °C for 1 h. The tubes were continually rotated in order to bathe the filter in lysis mixture. The manufacturer's protocol was then followed to extract fungal and viral DNA that was later 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. A total of 22 extractions were performed for each pathogen (44 total).

For PCR detection of *Bd*, total eukaryotic DNA was amplified using the EukA and 1195RE primer pair (all primer sequences are provided in Appendix 1) and ReadyMix Taq PCR Master Mix (Sigma) (Freeman *et al.* 2009a, Freeman *et al.* 2009b). 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 and 5.8S Chytr at a concentration of 900 nM each, the Minor Groove Binder (MGB) probe (Chytr MGB2) probe 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. Samples were considered positive for *Bd* if at least one of the triplicate reactions were positive.

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. Positive colonies were incubated at 95 °C for 10 min in 25 µl of sterile water and amplified using the M13F and M13R primer pair. PCR Products were then cloned into JM109 competent *E. coli* cells using the Promega pGEM-T Easy Vector System I. DNA from five positive colonies per sample was amplified using the M13F and M13R primer pair and was sequenced at the Molecular Core Research Facility at Idaho State University. Sequence analyses were performed using DNADynamo.

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 extracted viral

DNA, 12.5 µl 2 × iTaq Universal Probes Supermix (Bio-Rad), 300 nmol of forward rtMCP primer, 900 nmol reverse rtMCP primer, and 250 nmol of rtMCP probe. 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

Table 1. *Bd* and ranavirus eDNA qPCR results for water samples collected in the Gila and Mimbres drainages during the spring and fall of 2019 and the spring of 2020.

Sampling Period	Positive for *Bd*/ samples collected	Positive for ranavirus/ samples collected
Spring 2019	2/6	0/6
Fall 2019	0/14	3/14
Spring 2020	0/14	0/14

Overall prevalence of pathogens in the field and plant nurseries

Our results show that pathogens do occur in the Gila and Mimbres drainages as well as in plant nurseries (Table 1 and Table 2). For water samples taken from the field, 7.4% tested positive for either pathogen (5 of 68 samples tested; Table 1). Water samples from plant nurseries yielded a similar pathogen prevalence with 7.5% of the samples testing positive for either pathogen (3 of 40 positive; Table 2). Of the soil samples collected from plant nurseries 0% were positive for either pathogen (0 of 44 positive; Table 2).

Field sampling

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 the Gila Campground (1/3 qPCR replicates were positive; Figure 3). Ranavirus was not

Table 2. *Bd* and ranavirus eDNA test results for water and soil samples collected at four plant nurseries previously involved in riparian restoration projects.

Sample Site	Soil Samples Positive for Bd/samples collected	Soil Samples Positive for ranavirus/ samples collected	Water Samples Positive for Bd/ samples collected	Water Samples Positive for ranavirus/ samples collected
Plant Nursery 1	0/5	0/5	3/5	0/5
Plant Nursery 2	0/6	0/6	0/5	0/5
Plant Nursery 3	0/6	0/6	0/5	0/5
Plant Nursery 4	0/5	0/5	0/5	0/5

detected during the spring 2019 sampling (Figure 4). 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; Figure 5). Ranavirus was also detected on the Mimbres River at McKnight Rd (Figure 5). *Bd* was not detected in samples collected in the fall of 2019 (Figure 6). Finally, of the 14 locations

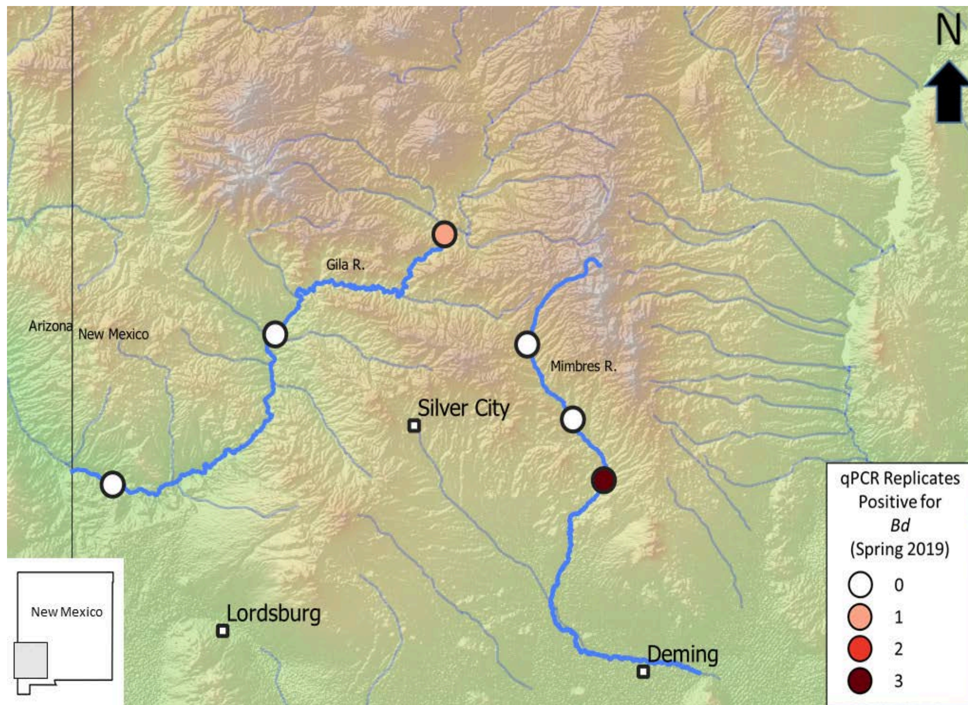

Figure 3. Map illustrating locations for eDNA water samples tested for *Bd* during the spring of 2019. Color indicates the number of positive qPCR replicates.

sampled during the spring 2020 (May), we did not detect either pathogen (Figure 7 and Figure 8).

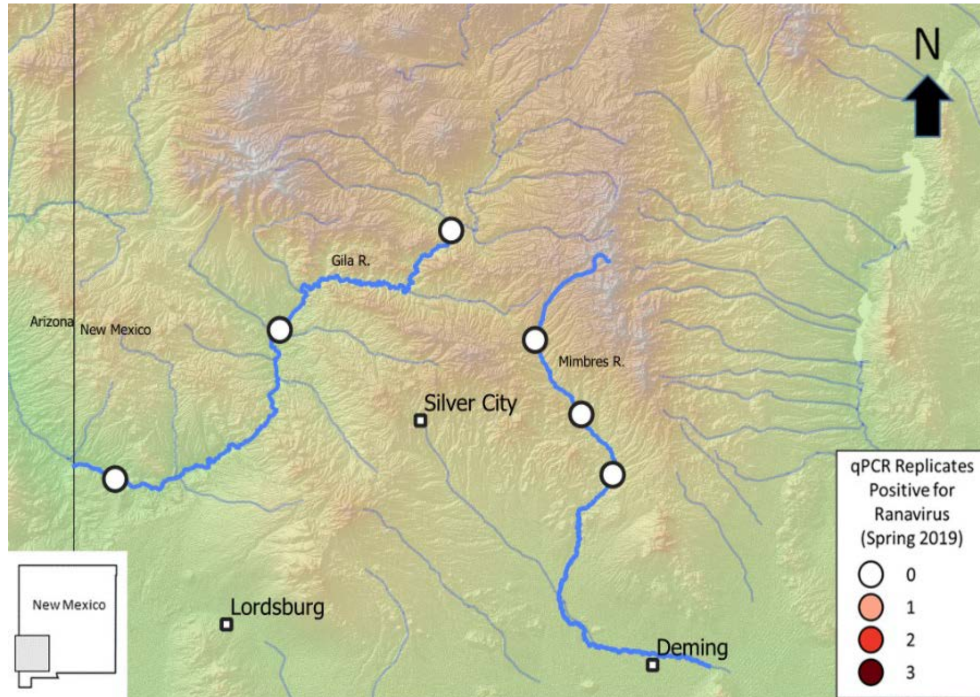

Figure 4. Map illustrating locations for eDNA water samples tested for ranavirus during the spring of 2019. Color indicates the number of positive qPCR replicates.

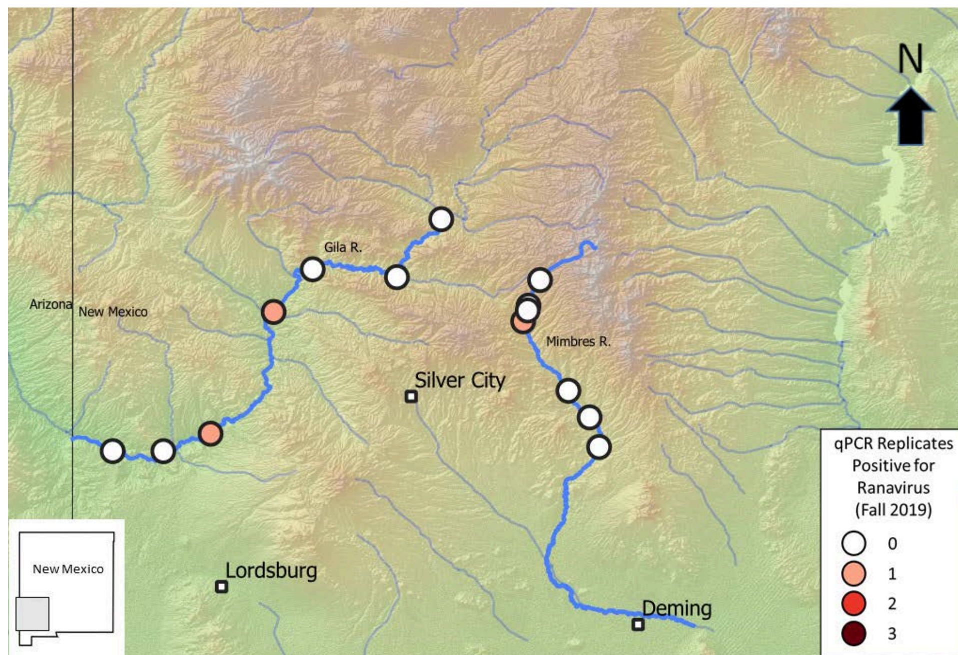

Figure 5. Map illustrating locations for eDNA water samples tested for ranavirus during the fall of 2019. Color indicates the number of positive qPCR replicates.

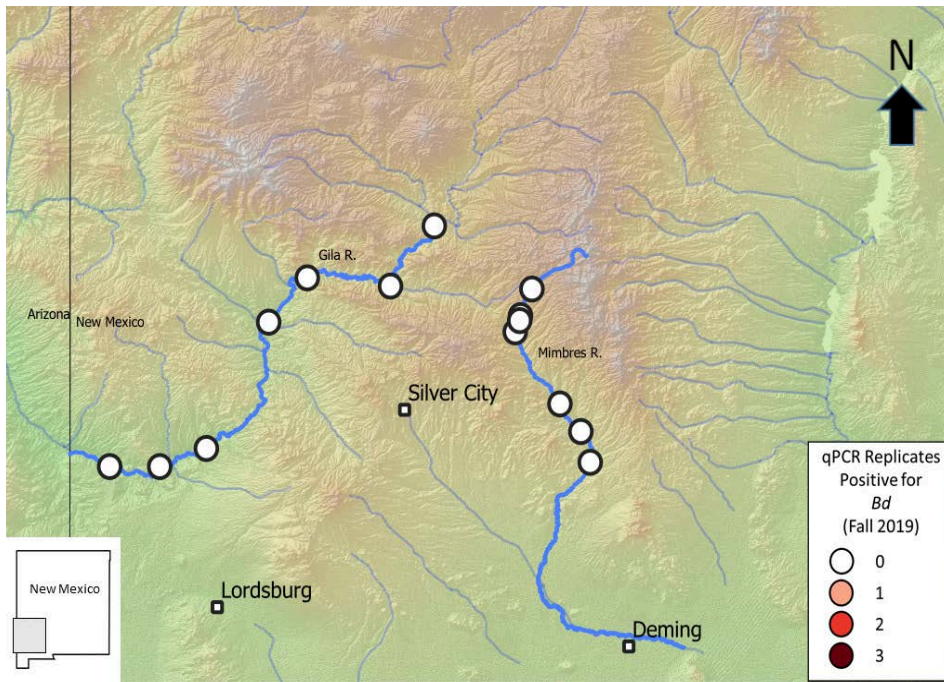

Figure 6. Map illustrating locations for eDNA water samples tested for *Bd* during the fall of 2019. Color indicates the number of positive qPCR replicates.

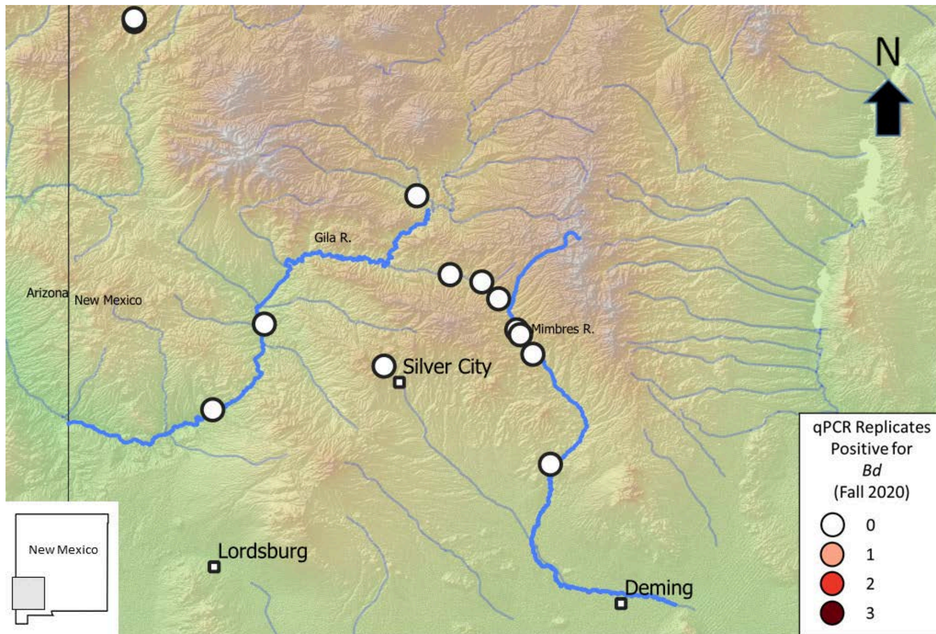

Figure 7. Map illustrating locations for eDNA water samples tested for *Bd* during the spring of 2020. Color indicates the number of positive qPCR replicates.

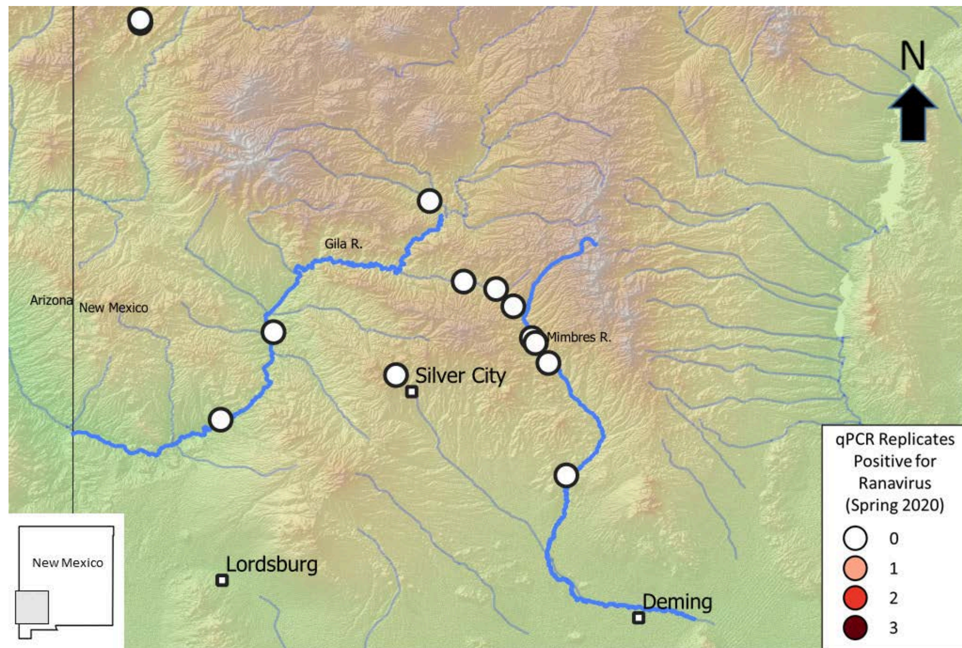

Figure 8. Map illustrating locations for eDNA water samples tested for ranavirus during the spring of 2020. Color indicates the number of positive qPCR replicates.

<u>Plant Nurseries</u>

Of the soil and water samples collected from plant nurseries, only *Bd* was detected in three water samples from Plant Nursery 1 (Table 2). These three positive water samples were from the wetbeds where *Spea* tadpoles were present (Figure 9).

Figure 9. Selected images of sampled material at Plant Nursery 1. Negative and positive symbols are used to indicate if the location tested positive for *Bd* with the number of “+” signs corresponding to the number of positive qPCR replicates.

Bd Isolate Identification

All isolate sequences are provided in Appendix 2. For each positive *Bd* sample, five clones were sequenced. Sequence analyses using DNADynamo revealed that each positive *Bd* sample consisted of the same isolate (i.e., all five clones were identical). The three *Bd* positive samples from plant nursery 1 were of the same isolate. The *Bd* positive water samples from the Gila River (Gila Campground) and the Mimbres River (near Faywood, NM; both collected in the spring of 2019) were unique from one another (95.97% identical). The positive sample from Plant Nursery 1 was also different from both the Gila and Mimbres samples (Plant Nursery 1 – Mimbres River samples are 94.67% identical; Plant Nursery 1 and Gila River samples are 93.96% identical). Overall similarity between sequences was 94.59%. We compared our sequenced results to the sequence database in GenBank and found the most similar entries were from *Bd* sequenced in China (Appendix 3). The Gila River sample was 95.27% similar to KX115393.1, the Mimbres River sample was 95.97% similar to KX115405.1 and the sample from Plant Nursery 1 sample was 98.64% similar to KX115392.1.

Discussion

The importance of riparian habitats and their vulnerability means that their restoration is critical for the management of populations of many wildlife species in the coming century (Smith et al 2014, Friberg *et al.* 2016). However, riparian restoration is not without risk. The focus of the current project was to evaluate the possibility of introducing aquatic vertebrate pathogens, especially novel strains of these pathogens, to riparian systems via planted flora. Introduction of plant pathogens by riparian restoration has previously been noted. For example, the plant pathogen *Phytophthora* occurs in plant nurseries and has been introduced into

restoration sites (Sims *et al.* 2019). Johnson and Speare (2005) speculated that *Bd* could be transmitted as a result of introducing plant materials from plant nurseries to the field. Ours is the first study to evaluate the risk of the introduction of the vertebrate pathogens *Bd* and ranavirus via riparian restoration projects.

We detected *Bd* in a wetbed at one plant nursery that was actively being used by amphibians (*Spea* tadpoles were present). We did not detect pathogens in the remainder of the collected soil or water samples from plant nurseries. Previous work suggests that *Bd* and ranaviruses are robust and capable of surviving in soil and water (Johnson and Speare 2003, Johnson and Speare 2005, Nazir *et al.* 2012). This suggests that while both pathogens may be capable of persisting in plant nurseries, they appear to be uncommon in the nursery setting. Furthermore, our results suggest that locations within a plant nursery should not be viewed equally with respect to the likelihood of pathogen prevalence. Wetbeds actively being used, or previously used, by amphibians should be viewed with concern. But by comparison, greenhouses often reach high temperatures during the spring and summer months (~32ºC). High temperatures may be less than ideal for the growth of *Bd* (Johnson et al 2003, Piotrowski *et al.* 2004) but may not inhibit ranavirus (Ariel *et al.* 2009, La Fauce *et al.* 2012). In addition, most of the plant nurseries sampled in this study used municipal water supplies for watering plants. We believe it unlikely that pathogens could survive the chlorination treatments performed in water treatment facilities. We also believe it unlikely that these pathogens are abundant in well water, given the lack of available hosts at the depth of the water table.

Bd was initially thought to be a monotypic lineage of recent origin. However, more recent research suggests that *Bd* is composed of multiple lineages (isolates) and is diverse geographically (Fisher *et al.* 2009, Farrer *et al.* 2011). Pathogenicity of *Bd* may vary significantly

depending on the pathogen's lineage and the susceptibility of the host (Berger *et al.* 2005). *Bd* isolates can vary at relatively small geographic scales (Morgan *et al.* 2007, Retallick and Miera 2007). Novel isolates may be introduced through human activities and have been linked to mortality events and extinctions globally (Schloegel *et al.* 2009). Our results suggest that *Bd* isolate diversity exists in both the field and plant nurseries in New Mexico: the *Bd* isolate found in the Mimbres was different from that found in the Gila and both differed from the isolate found in Plant Nursery 1. The sequenced markers from our study (Appendix 2) are most similar to GenBank entries collected in China (Appendix 3). These entries are associated with a publication (GenBank lists them as unpublished but these entries were not updated by the authors) in which the authors suggest that their sequenced isolates are native to China (Wang *et al.* 2018). We do not think that the isolates we detected originated in China. While the sequences are similar (95%-98%), they are not identical. In addition, recent work by Byrne *et al.* (2019) suggests that considerable undescribed lineage diversity exists for *Bd* at the global scale. However, our findings do confirm that *Bd* in Southwestern New Mexico is diverse. They further confirm that the risk of introducing pathogens via transplanting plant material from nurseries into the field is a real, however a modest, possibility and suggest that if pathogens are introduced, they could be of a different lineage than locally extant varieties of these pathogens, and may differ in their pathogenicity. Therefore, future projects should not simply consider the risk of introducing pathogens that are likely already present in the field but should also be aware of the possibility of the introduction of novel, potentially more lethal isolates of *Bd*. This information should be considered when planning future restoration projects.

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 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.* 2017, Hettyey *et al.* 2019). However, to our knowledge, no mitigation strategy has been developed for minimizing risk of introducing *Bd* and ranavirus during riparian restoration projects. Here we provide a few tactics for restoration project managers to consider when planning projects. First, our results suggest that plants grown in wetbeds should be avoided unless disinfection strategies are practiced (see below). This is particularly true if the wetbeds are actively being used by amphibians. However, we see no reason for managers to not work with plant nurseries that have wetbeds in their facilities. We detected *Bd* in wetbeds at Plant Nursery 1, but we did not detect pathogens in other water or soil samples taken from the facility. Second, we found evidence of lineage diversity among our detections of *Bd*. Thus, we suggest that collecting plant materials from within the same drainage as the restoration site would greatly minimize the risk of introducing a novel pathogen isolate. However, we recognize that this may not be an option in many circumstances. In these situations, we suggest prophylactically treating plant materials. Obviously, priority should be given to chemicals which are effective at removing target pathogens but do not harm the plant. For example, Virkon S (active ingredients: 20.4% Potassium peroxymonosulfate and 1.5% Sodium chloride) is a commercially-produced, broad-spectrum disinfectant that 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 during

tomato production. The authors applied Virkon S to tomato plants at a concentration of 2%, which is double the concentration recommended for disinfecting equipment to prevent *Bd* and ranavirus transmission. Thus, we expect plants such as cottonwoods and willow cuttings are likely to be able to tolerate Virkon S at least as well as tomato plants, particularly if a 1% concentration (as recommended for equipment decontamination) is applied. Our recommendation of Virkon S is intended as a starting point. Restoration project managers should work with greenhouse operators, consult the literature for new chemical treatments, and conduct experiments prior to broadscale application of any disinfectant on plants intended for use in restoration projects.

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Appendix 1

Primer and probe sequences used for *Bd* and ranavirus qPCR detection, cloning, and sequencing.

EukA: 5'-AACCTGGTTGATCCTGCCAGT-3'

1195RE: 5'-GGGVATCACAGACCTG-3'

ITS1-3 Chytr: 5'-CCTTGATATAATACAGTGTGCCATATGTC-3'

5.8S Chytr: 5'-AGCCAAGAGATCCGTTGTCAAA-3'

Chytr MGB2: 5'-6FAM CGAGTCGAACAAAAT MGBNFQ-3'

rtMCP-F: 5'-ACACCACCGCCCAAAAGTAC-3'

rtMCP-R: 5'-CCGTTCATGATGCGGATAATG-3'

rtMCP: 5'-FAM CCTCATCGTTCTGGCCATCAACCAC TAMRA-3

M13F: 5'-GTAAAACGACGGCCAG-3'

M13R: 5'-CAGGAAACAGCTATGAC-3'

Appendix 2

5.8S ribosomal RNA gene sequences for positive *Bd* detections.

<u>Gila Campground Sequence</u>

CCTTGATATAATACAGTGTGCCATATGTCACGAGTCGAACAAAATTTATTTATTTTTT

CGACAAATTTAATTGAAATGAATGTTAATTTAATAAAAAATTGAAAATAAATATTAA

AAACAACTTTTGACAACGGATCTCTTGGCT

<u>Mimbres Faywood DNA Sequence</u>

CCTTGATATAATACAGTGTGCCATATGTCACGAGTCGAACAAAATTTATTTATTTTTT

CGACAAATTAATTGGAAATTGAATTAATTTATGAAAAAAATGTGAAATTTAAATATT

AAAAACAACTTTTGACAACGGATCTCTTGGCT

<u>Wet Bed DNA Sequence</u>

CCTTGATATAATACAGTGTGCCATATGTCACGAGTCGAACAAAATTTATTTGTTTTTT

CGACAAATTTATTGGAAATTGAATAATTTAATTGAAAAATAATTGAAAATAAATTTA

TTAAAAACAACTTTTGACAACGGATCTCTTGGCT

Appendix 3

The links below are to GenBank entries for 5.8S ribosomal RNA gene sequences (*Bd*) which are most similar to those found in the current study.

KX115393.1

KX115405.1

KX115392.1