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**LEVERAGING HERBARIA SPECIMENS: A NEW 3D IMAGING PROTOCOL AND
ANALYSES OF DIVERSITY OF WEST VIRGINIA ROSIDS**

A thesis submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Master of Science

In

Biological Sciences

by

Zachary Scott Shamblin

Approved by

Dr. Pamela Puppo, Committee Chairperson

Dr. Anne Axel

Dr. Kyle Palmquist

Dr. Herman Mays

Marshall University
December 2022

APPROVAL OF THESIS

We, the faculty supervising the work of Zachary Shamblin, affirm that the thesis, *Leveraging Herbaria Specimens: A new 3D Imaging Protocol and Analyses of Diversity of West Virginia Rosids*, meets the high academic standards for original scholarship and creative work established by the Department of Biological Sciences and the College of Science. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.



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ABSTRACT

Herbaria are repositories of plant natural history, whose data can provide insights into the life histories of plant taxa over space and time. Recent initiatives to digitize herbarium data have made studies over broad geographic and taxonomic extents possible, with the ability of scientists to access data from large databases of herbaria. Most of the digitization initiatives have focused on flat 2D herbaria sheets, but herbaria also include 3D collections, such as fungi, mosses, lichens, and dry fruits. In Chapter 1 we propose an easy and cost-effective workflow that uses free photogrammetry software to produce 3D images of 3D herbaria specimens. We tested four free to use photogrammetry software and created 3D images of five types of 3D specimens: a fungus, a moss, a lichen, a pinecone, and a compound fruit. With our workflow using equipment already present in most herbarium digitization stations, we produced 3D specimens in less than an hour. The software 3DF Zephyr Free gave the best results across each specimen type. Our workflow has provided an easy and low-cost method of producing 3D images that can be used to digitize 3D specimens in a similar manner to 2D herbaria sheets. In Chapter 2 we used herbaria data to analyze the spatial patterns of diversity of a major clade of plants, the Rosids, that contain most hardwood trees in West Virginia, and are the dominant plant species in the state. We used phylogenetic diversity to assess the diversity of this clade across the Level IV Ecoregions of West Virginia. Phylogenetic diversity (PD) is a metric that incorporates the evolutionary history of an area of interest. Ecoregions are defined by the Environmental Protection Agency (EPA) as areas of similar habitat, climate, and species composition. Our results indicated high PD in the eastern portion of the state in the Monongahela National Forest and low PD in the southern portion of the state in the Dissected Appalachian Plateau ecoregion. Analyses such as ours are

important for informing conservation priorities, as they identify specific areas that are more biodiverse.

CHAPTER 1

DIGITIZING 3D HERBARIUM COLLECTIONS USING FREE, OPEN-SOURCE 3D RECONSTRUCTION SOFTWARE

INTRODUCTION

Over the past decade, the digitization of natural history collections has been an invaluable tool in biodiversity studies. Specifically, specimens found in herbaria have been particularly useful in plant diversity research. With millions of plant specimens digitized, and label data transcribed, researchers can reliably assess distribution of specific taxa and communities, evaluate potential shifts in timing of life history processes over time (Soltis 2017), calculate phylogenetic diversity of plant communities (Thornhill et al. 2017; Spalink et al. 2018a; Allen et al. 2019), and even use machine learning to distinguish morphologically similar taxa among a database of digitized herbaria specimens (Schuettpelz et al. 2017).

Massive digitization efforts such as the Southeastern Regional Network of Expertise (SERNEC 2022), the Consortium of Midwest Herbaria (2022), and others, have produced hundreds of thousands of 2D images of herbarium specimens. However, herbaria also contain three dimensional specimens, such as, but not limited to: fungi, dried fruits, mosses, and lichens. These 3D specimens have been included to some extent in digitization initiatives, such as the Mycology Collections Portal (Miller and Bates 2017) or the HerbIMI (2022) but using the same photograph setting for 2D specimens or only including label information. Visualizing these specimens in three dimensions may provide opportunities for morphological analyses, where two dimensional images exclude major types of phenotypic data, such as shape of organs (Cardini

2014). Also, three dimensional images of specimens can be useful in species identification, when detailed morphology is required (Gabelaia et al. 2018; Giacomini et al. 2019). Prior approaches to 3D imaging natural history collections have involved tomography and laser scanning (Mathys et al. 2013), which are cost prohibitive and require intensive training to use properly.

Photogrammetry, the process in which a 3D model of an object is created by taking multiple pictures of the object from multiple angles, has also been used to image archaeological collections, and to some extent, natural history collections (Douglass et al. 2015; Tsuboi et al. 2020). For example, Medina et al. (2020) incorporated 288 photos to generate a 3D image of one bird specimen, and Nguyen et al. (2014) incorporated 10-17 GB worth of images for a detailed 3D model of an insect. Both studies are similar, utilizing a DSLR (digital single-lens reflex) camera for image capture. These methods of photogrammetry successfully generated 3D models that provide detailed representations useful for natural history studies. However, the cost of the photogrammetry software used, together with the hardware and skill required to produce such images may be unaffordable to many botanical institutions to effectively image large collections of 3D specimens.

Here we propose a low cost and straightforward workflow for digitizing three-dimensional specimens using equipment already available in most herbaria digitization stations such as a camera and a light box. Using free photogrammetry software and 40-50 images, we are able to produce 3D images of 3D herbarium specimens in approximately 50 minutes per specimen. We tested these reconstructions using five specimens, a fungus, a moss, a lichen, a pinecone, and a compound dry fruit (Appendix B). We compare four photogrammetry software based upon output obtained and ease of use and make an overview of the different alternatives to view and share the 3D images produced.

MATERIALS AND METHODS

Setup and Workflow

Our setup made use of a digitization station present in the Marshall University Herbarium (MUHW) composed of a lightbox, a camera, and a camera stand (Fig. 1). The camera was a Nikon D610 with a 60 mm lens (Nikon 2013). We purchased an inexpensive, lightweight 50-inch tripod for this project (Amazon Basics, ca. \$18 on Amazon.com, Appendix C). While a tripod is recommended, it is not necessary, and suitable images may be obtained via a free hand method. The lightbox in the herbarium (Or Technologies 2005) was also used, and we purchased an 11-inch white rotating cake stand (Kootec brand, ca. \$11 on Amazon.com, Appendix C) to use as rotating tray. The specimen was placed on the rotating tray and rotated approximately 30° for every shot, until a 360° field of view was obtained. This was done with the camera facing the specimen at 0° and then again at approximately 45° above the specimen. Then close up shots of fine details, were taken to provide more detailed texturing. For the fungus, pinecone, and compound fruit, we also used a clear acrylic stand (Artliving brand, ca. \$10 on Amazon.com, Appendix C) to elevate the specimens from the rotating tray and facilitate photographing (Fig. 1). As with the tripod, the rotating tray or acrylic stand are not necessary to produce suitable images for meshing but may save time imaging and also produce more consistent results across various specimens. The lightbox was set to provide even diffuse lighting from all angles on the object. Any shadows present in the photographs will appear in the final mesh result, so it is recommended to adjust lighting to minimize shadows. Also, it is important that the specimen be in focus and at the center of the image. Any object, such as the rotating plate or acrylic stand, must be inconspicuous, as well as any grid marking. Grid paper was placed under the specimen

to assist the mesh processing software in determining the location of each camera or shot, with respect to the specimen. Specific camera settings, such as focal length and aperture were manually adjusted using the tether capture function of Adobe Photoshop Lightroom Classic (Adobe Inc. 2021). Settings followed the 3D photogrammetry workflow from Haines (2019): ISO 160, aperture (f) $f11$, shutter speed $1/80$, and automatic white balance. A total of 40-50 images were taken per specimen. These were exported into the photogrammetry software and a sparse point cloud was generated using the initial “structure from motion” setting. A sparse point cloud is a rough approximation of the object, created from the multiple images. The next steps are an automated or manual pipeline that first produces a dense point cloud from the sparse point cloud and then produces an untextured 3D model (Appendix D). The end goal of the process is a textured mesh, which may be exported as a 3D object (.obj). The textured mesh may be viewed with the default 3D Viewer application in Windows.



Figure 1. General set-up of imaging station

The specimen is placed on a turntable in the lightbox and the camera is set to capture images as the specimen is rotated. An acrylic stand is used to elevate some specimens to facilitate photographing.

Software

In this study, we compared four photogrammetry software: Meshroom (2022), 3DF Zephyr Free (2022), ColMap (2022), and Regard3D (2022) (Table 1). More software are available, but these four were chosen due to being free to use and having minimal hardware requirements. While these four software can produce 3D models, not all can produce a textured mesh, a rendered 3D model with detailed surface features. ColMap may only produce an untextured 3D model that lacks detailed surface features, which must then be exported to a different software capable of textured mesh processing. This of course is a significant disadvantage that limits the utility of this software. Each of the listed software come with certain caveats that must be considered. Meshroom requires hardware with a Compute Unified Device Architecture (CUDA) enabled GPU to perform mesh processing. CUDA is an architecture developed by NVIDIA (Santa Clara, California, US) that uses both the GPU and CPU of a system to perform faster processing of intensive tasks such as 3D rendering. 3DF Zephyr Free has a 50-image limit for each workflow, which can be circumvented only by purchasing the Lite version (one-time payment of \$187 for up to 500 images) or Full version (one-time payment of \$4,902, with unlimited images) of the software. Regard3D is not as intuitive and less user friendly than the other three software.

Software	GPU Requirements	In Software Textured Mesh Processing	Output File Format	Example Mesh	URL
3DF Zephyr Free	No specific requirements	yes	.obj	Fig. 2A Fig. 3A Fig. 4A Fig. 5A Fig. 6A	https://www.3dflow.net/
Meshroom	CUDA Required	yes	.ply, .obj	Fig. 2B Fig. 5B Fig. 6B	https://alicevision.org/
Regard3D	No specific requirements	yes	.ply, .obj	Fig. 2C Fig. 3B Fig. 4B Fig. 5C Fig. 6C	https://www.regard3d.org/
ColMap	CUDA and non-CUDA available	no	.ply	Fig. 2D Fig. 3C Fig. 4C Fig. 6D	https://colmap.github.io/

Table 1. Comparison of software based upon GPU requirements

Comparison of software based upon GPU requirements; whether or not the software has in house textured mesh processing, output file format, and visual comparison of final meshes.

The use of CUDA compatible graphics cards in mesh processing is recommended due to specific requirements of software and due to a more rapid processing time. However, software exist that can perform mesh processing without the use of CUDA enabled graphics cards.

Whether or not a graphics card is CUDA capable may be checked in Windows (see <https://docs.nvidia.com/cuda/cuda-installation-guide-microsoft-windows/index.html>). Current iterations of CUDA drivers do not support MacOS; however, archived versions of CUDA drivers for MacOS may be downloaded from NVIDIA: <https://www.nvidia.com/en-us/drivers/cuda/mac-driver-archive/>.

For sharing with other viewers, the 3D object may be embedded in a webpage or any format in the Microsoft Office 365 Suite. However, the action of webpage embedding comes with the necessity of being fluent in webpage design to embed the 3D Object or the use of a service such as Sketchfab (2022) which allows the user to upload and edit a 3D object, redeem a code, and only then copied/pasted into the webpage HTML. The free version of this service has a maximum of 10 uploads per month and the basic paid plan for organizations is \$948 yearly. Tutorials are available on the web for a “DIY” approach to embedding 3D objects, but a familiarity in HTML and CSS is recommended. Another important consideration is the file size of 3D objects and the associated images used to construct them. For example, the 3D file for Figure 2 was 18 MB and the image folder was 125 MB. It will be necessary for each institution to have sufficient storage space, either in physical hard drives or in cloud data storage.

RESULTS

In this study we assessed the usefulness of four free photogrammetry software (Meshroom, 3DF Zephyr Free, ColMap, and Regard3D) to obtain 3-D reconstructions of five types of 3-D herbarium specimens: a fungus, a moss, a lichen, a pinecone, and a compound dry fruit. The time it took for imaging a single specimen (40-50 photos) was approximately 15 to 20 minutes, and the average time to render a textured mesh was ca. 30 minutes. The latter would vary depending on the software used, GPU, and CPU capabilities. For this study, we used a

Windows PC with an Intel i5 processor, 16 GB RAM, no GPU, which took 30 minutes to render this textured mesh. Additional editing to eliminate unwanted features from the mesh, such as background and the stand in which the object sits, were factored into the total time it takes to render a completed mesh. Depending on the software used, it may be necessary to edit the mesh in a separate software. From the four programs tested, the only mesh processing software with in-software mesh editing was 3DF Zephyr Free. For the other three software, a separate mesh editing software, MeshLab (2022), was used. There are a number of possible free mesh editing software, but this also comes with additional challenges such as learning how to operate them. MeshLab was used due to ease of use and the large amount of support documentation.

It is important to note that the same set of 40-50 images did not yield the same results across the four software compared (see Figs. 2-6). For example, in the photoset used for Figure 2, a textured mesh was created from 48 out of 50 key points. This means the software was able to identify similar features in 48 out of the 50 photos to reconstruct a 3D image. In Meshroom, many of the images were unable to be used, yielding a textured mesh with the back side of the object missing or failing to reconstruct the object altogether (Figs. 3 and 4). The solution to this would be to incorporate more images into the reconstruction, which will ultimately yield a more detailed mesh, but would increase imaging time and the storage space needed for the images.

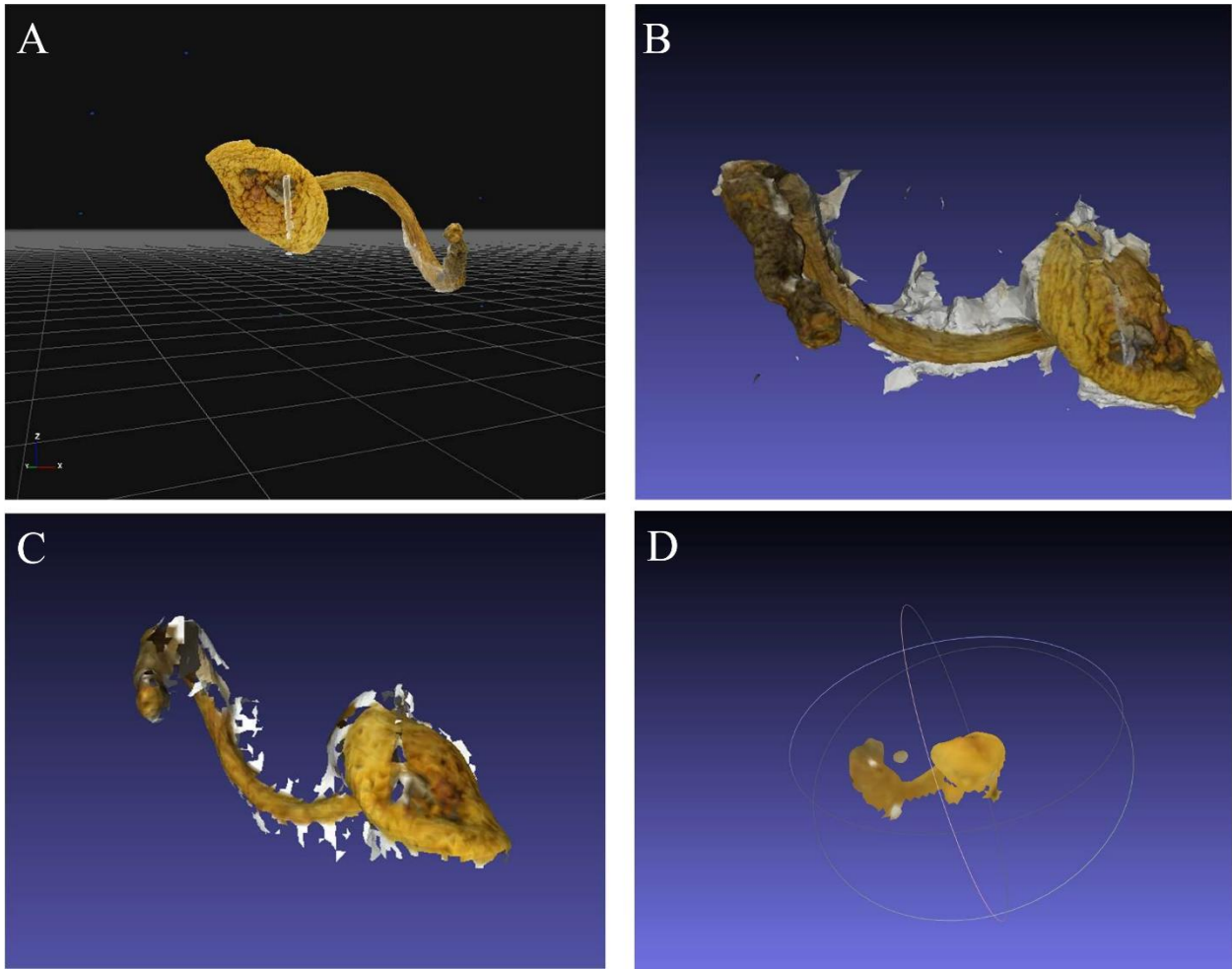


Figure 2. Textured mesh of a *Cortinarius* sp. fungi generated using the four photogrammetry software tested in this protocol

A. 3DF Zephyr Free; B. Meshroom; C. Regard3D; D. ColMap.

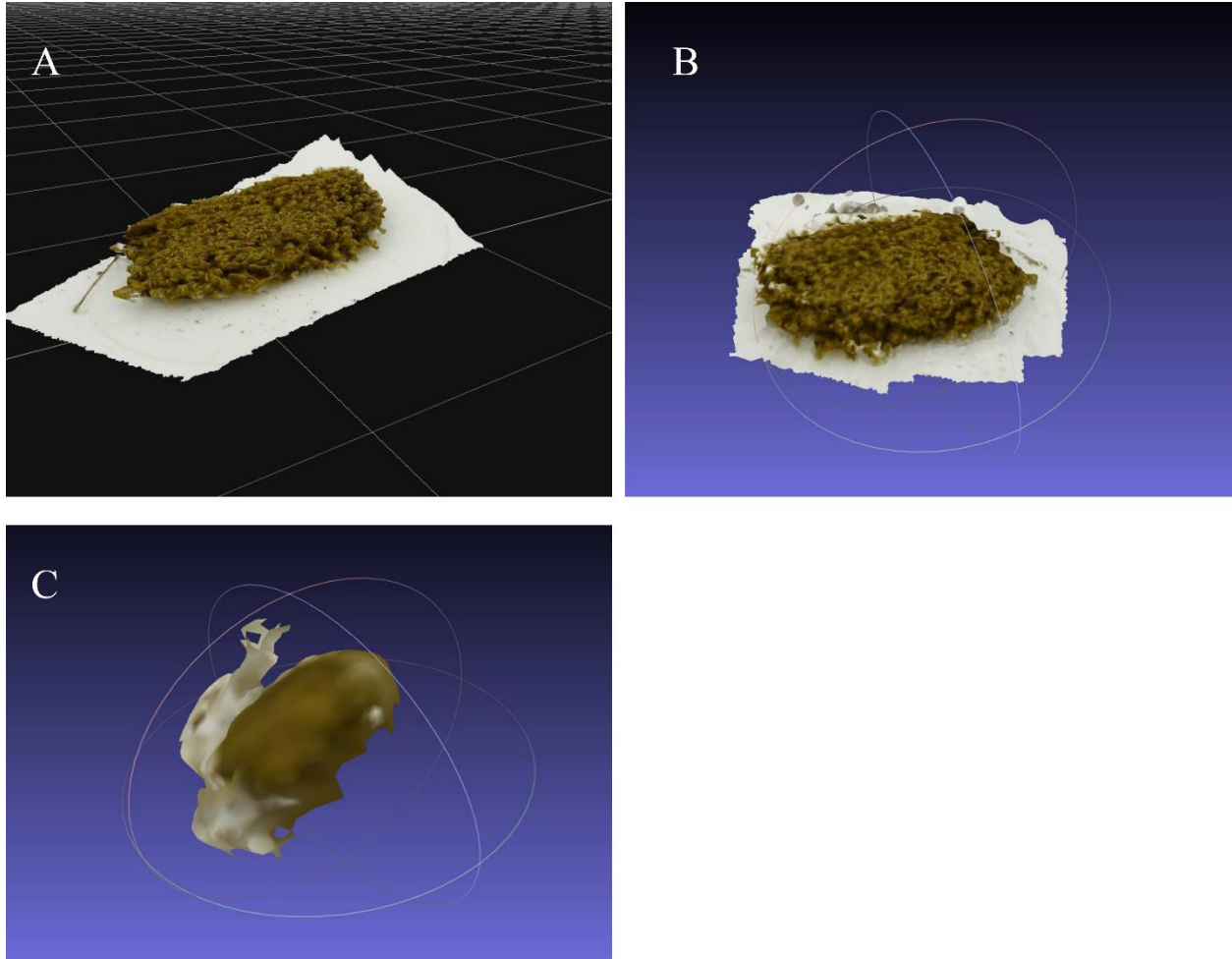


Figure 3. Textured mesh of a moss, *Dicranum fulvum*, generated using three photogrammetry software

A. 3DF Zephyr Free; B. Regard3D; C. ColMap. Meshroom software failed to generate a textured mesh.

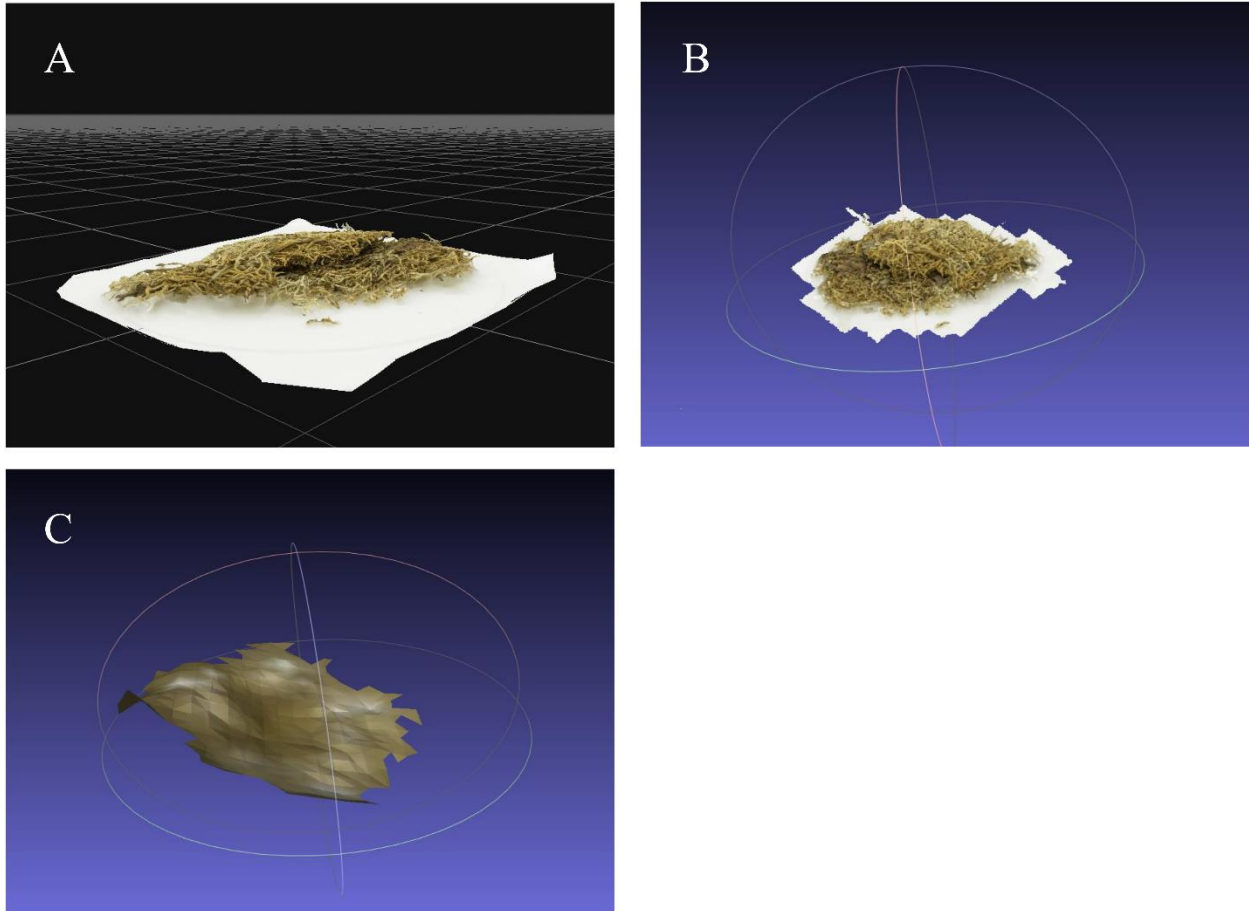


Figure 4. Textured mesh of a lichen, *Cladonia furcata*, generated using three photogrammetry software

A. 3DF Zephyr Free; B. Regard3D; C. ColMap. Meshroom software failed to generate a textured mesh.

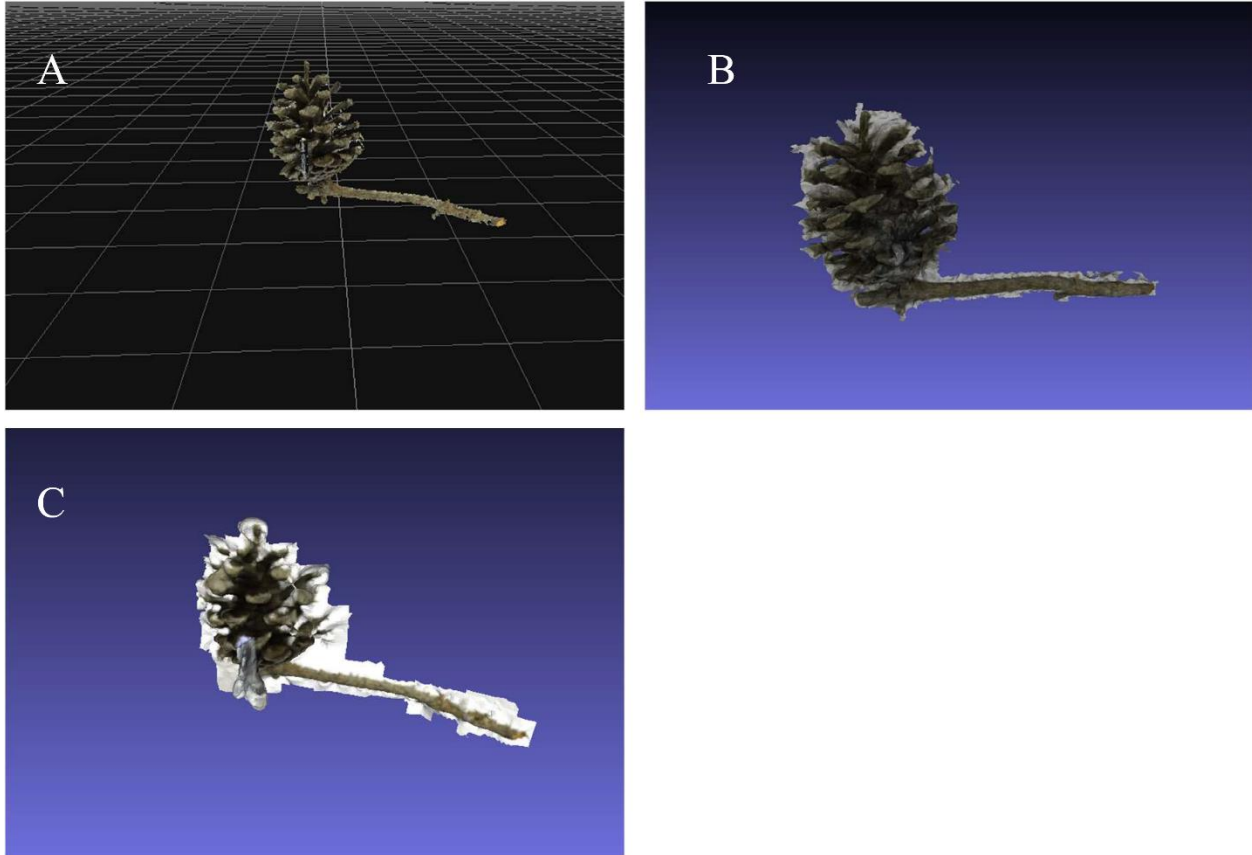


Figure 5. Textured mesh of a pinecone from *Pinus virginiana*, generated using three photogrammetry software

A. 3DF Zephyr Free; B. Meshroom; C. Regard3D. ColMap software failed to generate a viable point cloud to generate a textured mesh.

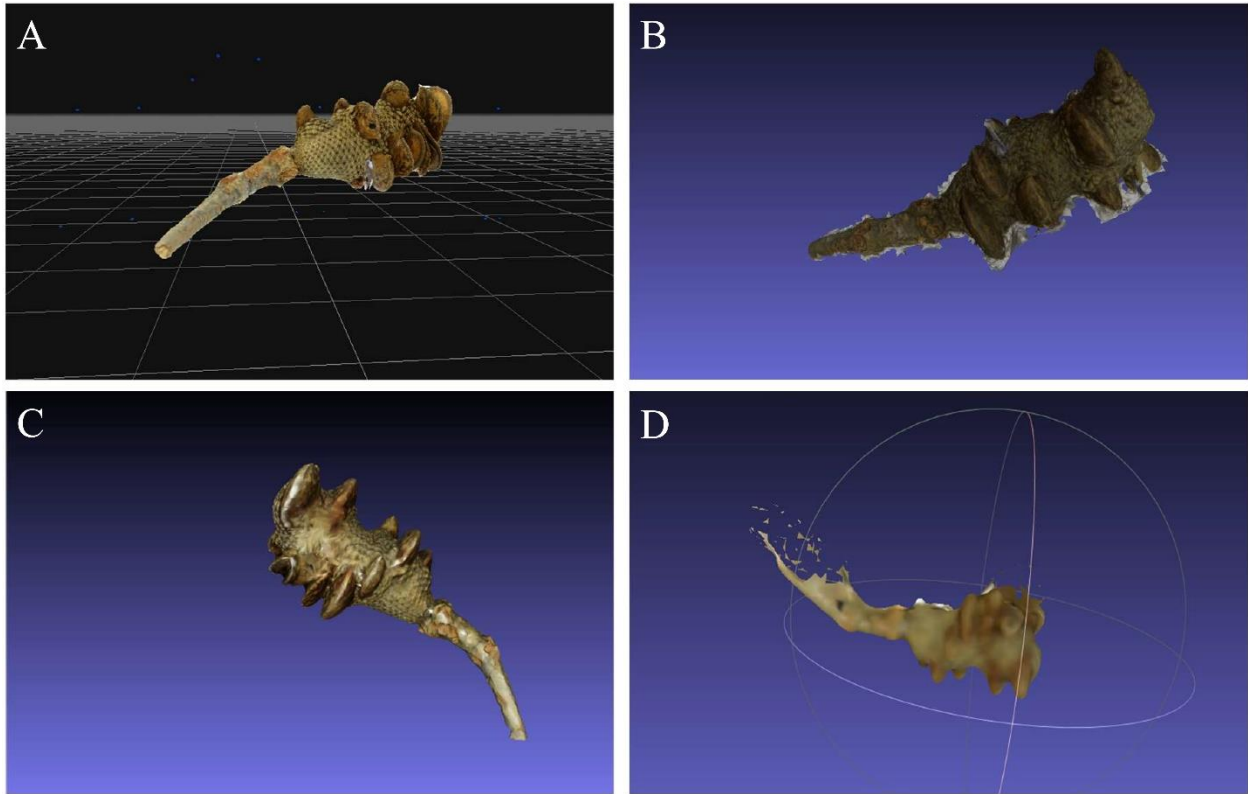


Figure 6. Textured mesh of a compound fruit of *Banksia* sp. generated using the four photogrammetry software

A. 3DF Zephyr Free; B. Meshroom; C. Regard3D; D. ColMap.

From the four software tested, 3DF Zephyr Free was the only software capable of incorporating 50 images or less to create a detailed textured mesh with minimal hardware requirements for each of the specimens tested (Figs. 2-6). Also, 3DF Zephyr Free does not require extra software to view or edit meshes. Appendix E provides step-by-step instructions to produce 3D images using the 3DF Zephyr Free software. However, while the photogrammetry software identified and incorporated most of the images from the fungus and compound fruit specimens, many of the images for the lichen and moss specimens were left out of the mesh

generation. We also attempted to create a textured mesh of a pinecone, but this ultimately failed, and only half of the specimen was rendered. This may be due to the pinecone being a radially symmetrical object and therefore the software was unable to differentiate the front and back of the specimen. Objects with homogeneous features, such as pinecones, mosses, and lichens, constitute a challenge to 3D reconstruction software, as these objects lack the distinctive features that aid in orienting the cameras during scene reconstruction. Sometimes there might be holes in the 3D reconstruction if the object is sitting on a surface such as the rotating tray. This is why a clear acrylic stand might be used to elevate the specimen and facilitate imaging of the bottom part. Still, it may not be possible to completely remove these stands from the 3D image without removing part of the specimen too.

DISCUSSION

The protocol described here, provides a low-cost and effective method of imaging 3D specimens, using digitization equipment already available in most herbaria. Free-to-use photogrammetry software, such as 3DF Zephyr Free, can construct detailed textured meshes of 3D specimens without the need for purchasing expensive yearly subscriptions to commercial software. As the digitization of flat herbaria sheets has facilitated the mining of data for broad analyses, the digitization of 3D herbaria specimens has the potential to be useful in a similar manner.

Compared to previously published methodologies for imaging 3D specimens, our workflow is both less time consuming and less resource intensive. Ngyuen et al. (2014), devised 3D models of insects using images taken at 144 different angles and at 31 different distances. Before implementing a process in which the images are stacked to reduce storage, 10-17 gigabytes of images were generated for a single specimen. Similarly, Medina et al. (2020),

constructed 3D objects from 288 photos, taken at 3 different angles, for a single specimen. Our methodology used 40-50 images to reconstruct a 3D model, which significantly decreases the time spent per specimen, also requiring significantly less hard drive space to store images.

The use of these photogrammetry software might be more suitable for 3D specimens such as fungi or dry fruits that have a bilateral symmetry or are asymmetrical, as several of these software have difficulties reconstructing objects with radial symmetry or that have repetitive patterns such as pinecones, lichens, and mosses. For these specimens, classical 2D photography might still be the best approach available.

CHAPTER 2

ASSESSING PHYLOGENETIC DIVERSITY OF WEST VIRGINIA ROSIDS WITH HERBARIA DATA

INTRODUCTION

Habitat destruction and climate change are the two greatest threats to plant biodiversity globally (Brooks et al. 2002; Dawson et al. 2011). Habitat loss due to changes in land use coupled with the effects of global climate change are resulting in shifts in spatial patterns of biodiversity and decreasing the amount of suitable habitat for plants (Bellard et al. 2012; Mantyka et al. 2012; Corlett 2016). With the potential loss of diversity, it is important to understand the spatial distribution of taxa as this may provide key insights into areas rich in species and areas in which biodiversity is declining or is expected to decline (Greve et al. 2016). This in turn would allow for the adequate allocation of resources to conserve species that may be threatened with habitat loss. Also, this would be important in conserving regions in which great diversity of species already exists.

Recent studies assessing biodiversity of particular regions, have also considered the evolutionary history of the area (Mishler et al. 2014; Thornhill et al. 2018; Spalink et al. 2018a; Spalink et al. 2018b; Allen et al. 2019). Evolutionary history is relevant in biodiversity studies because the formation of plant assemblages is informed by the circumstances under which taxa have diversified in their associated habitats to form the biodiversity that is present today (Mishler 2010). Biodiversity analyses that incorporate phylogenies become more than a simple count of species and incorporate the aspects of unique evolutionary history for an area. One of the key metrics used in biodiversity studies is Faith's phylogenetic diversity (PD) (Faith 1992). PD is a

sum of the branch lengths within a phylogeny and can be seen as a method of assessing the breadth of traits within a site of interest considering evolutionary history. Higher PD would indicate a wider breadth of niches that taxa fill in an area. Higher PD is also correlated with the overall functioning and stability of ecosystems (Cardinale et al. 2012; Cadotte et al. 2012). The more niches are filled by native taxa, the more these niches might be protected against invasive species as an “insurance effect” (Hector et al. 2010). Also, greater diversity may contribute to resistance and quicker recovery after a disturbance (Isbell et al. 2015). Another important metric of biodiversity is mean pairwise distance (MPD) (Webb et al. 2008). MPD measures the relatedness of taxa in a particular study site (Kembell et al. 2010; Webb et al. 2008). While PD considers the depth of evolutionary history and is a proxy for taxa richness, MPD considers breadth of evolutionary history and is indicative of niches filled by taxa of a particular study site. MPD is often used in conjunction with PD because while both metrics provide information on the evolutionary diversity of taxa, each provide different contexts to evolutionary diversity. PD is a richness metric, measuring the abundance of unique taxa, while MPD is a divergence metric, measuring the evolutionary distinctiveness of assemblages as a whole (Tucker et al. 2017).

The Appalachians encompass a broad geographic region of mountains and associated plateaus and hills in the eastern United States and Canada that spans from Newfoundland through northern Alabama. The Appalachian Mountains were formed about 480 Mya by the collision of the Laurentian and Gondwanan plates (Hopper et al. 2017). These mountains are an area of high plant diversity in the U.S., as Central Appalachia served as a refugia for different taxa during glaciation events in the Northern Hemisphere (i.e., Walker et al. 2009; Herman and Bouzat 2016). West Virginia is a state located completely within the Appalachia region, and its biodiversity is poorly studied. Moreover, since the Appalachians contain deposits of both

anthracite and bituminous coal, mining activity in the state has led to significant habitat loss, especially in central Appalachia (Palmer et al. 2010; Wickham et al. 2013). Assessing the distribution of plant diversity in West Virginia is important for basic science and also to inform conservation efforts and prevent the loss of more diversity to human land use activities such as mining.

In West Virginia, the majority of hardwood tree species such as oaks, walnuts, beeches, maples, and elms are part of an important group of plants known as the Rosids (Weakley 2022). The Rosids are a broad clade of approximately 70,000 plant species worldwide that comprise 25% of the total Angiosperm diversity (Wang et al. 2009). This broad clade is composed of hundreds of species of herbaceous plants, shrubs, and trees. Members of this clade are also foundational to the structure and function of forests in West Virginia, like oak species (*Quercus sp. L.*), that are important in the fire regimes of these forests (Van Gundy and Morin 2021). Some plant orders included in this clade such as Fagales provide an important food source to the fauna in the state, including acorns (*Quercus sp.*), walnuts (*Juglans sp. L.*), and hickory nuts (*Carya sp. Nutt.*) (Morin et al. 2016), or Fabales, the nitrogen fixing order, whose members have formed a symbiosis with nitrogen fixing bacteria that make nitrogen available to other associated plants (Koenen et al. 2019). Rosid species in the temperate zone experienced rapid diversification during the last glacial period, 15 to 10 Mya and the southeastern United States may have served as a refugia for these rapidly diversifying Rosid species, which subsequently dispersed as glaciers retreated (Sun et al. 2020).

In this study, we assess the spatial patterns of phylogenetic diversity of the Rosids clade in West Virginia using herbaria occurrence records, mostly from the three largest herbaria in the state: West Virginia University, Marshall University, and Wesleyan College. Our aim is to

identify areas where high plant diversity exists. For this, we inferred a phylogeny of native Rosid taxa and calculated the spatial patterns of Phylogenetic Diversity (PD) and Mean Phylogenetic Diversity (MPD) in West Virginia. We also identify which orders within the Rosids are the most diverse in the state.

MATERIALS AND METHODS

Study Area

The area of study encompasses the state of West Virginia, U.S., from 37.2 to 40.6 N and from 77.7 to 82.6 E, with an area of approximately 62,755 km². To classify the taxa in this study, we used the Level IV Ecoregions as these are the finest level of Ecoregions in the current Environmental Protection Agency (EPA) framework (Fig. 7). The EPA lists 15 Level IV ecoregions of varying area for the state of West Virginia (Omernik and Griffith 2014). The two largest ecoregions are the Forested Hills and Mountains Ecoregion and the Dissected Appalachian Plateau, which encompass the eastern and southwestern portion of the state, respectively. The Forested Hills and Mountains Ecoregion contains federally protected lands such as New River Gorge National Park and the larger Monongahela National Forest. The Dissected Appalachian Plateau Ecoregion contains mostly private land and land used for commercial mining and logging. In the eastern portion of the state, along the border with Virginia and in the eastern panhandle, there are smaller Ridge and Valley subtype ecoregions. These ecoregions are dominated by limestone, shale, sandstone, and dolomite substrate (Woods et al. 1996).

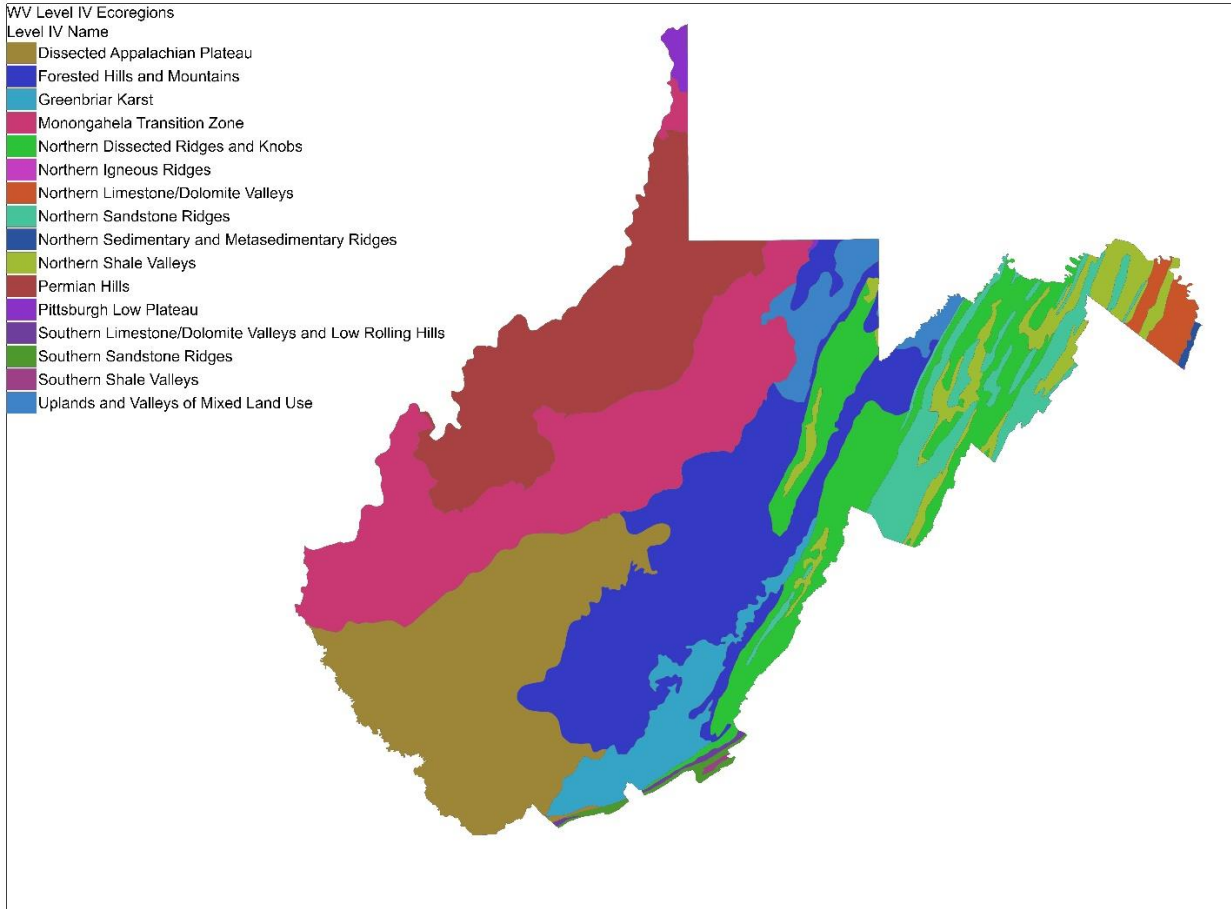


Figure 7. Level IV Ecoregions of West Virginia

Level IV Ecoregions of West Virginia derived from Omernik and Griffith (2014).

Study Specific Phylogeny

A list of Rosid species native to West Virginia was obtained from Weakley’s Flora of the Southeastern United States: West Virginia (Weakley 2022). While members of the clade Eurosids, or the true Rosids were considered in the biodiversity analyses, members of the Superrosids clade were considered for the study specific phylogeny. The Superrosids clade

contains the Eurosids, Vitales, and Saxifragales as an outgroup. With this list, there were approximately 646 distinct taxa that occur within West Virginia. From these, 360 were native and were used to construct the phylogeny, as we wanted to focus primarily on the diversity of native species. Whether or not a taxon was native was determined using Weakley's Flora. Only species were included in the phylogeny, no taxonomic ranking below species was considered.

A study specific phylogeny was inferred with the list of 360 native West Virginia Rosids species using the Phylomatic function within the software Phylocom (Webb 2008). Phylomatic is a function that creates a study specific phylogeny by parsing the species of interest from a larger tree. For this study, we used a mega tree of all Angiosperm genera (Slik et al. 2018) available in Phylocom. This input mega tree lacks branch lengths, so we produced an ultrametric tree to calculate phylogenetic diversity by generating a chronogram via the 'bladj' algorithm in Phylocom. The algorithm generates a chronogram by calibrating the branch lengths of an input phylogenetic tree with an 'ages' file, which contains the estimated ages of nodes within the tree. The default ages file contained in Phylocom, is derived from the ages of nodes within the Angiosperms obtained by Wikiström et al. (2001). The root node for our tree was assigned as 'subrosid,' which has an estimated age of 121 My.

Phylogenetic Diversity Metrics

In order to calculate diversity metrics, 20,000 occurrences of Rosid specimens from West Virginia were obtained from the three main Herbaria in the state. Approximately 5,000 specimens were obtained from the Southeastern Regional Network of Expertise (SERNEC) database (sernecportal.org), most from the Marshall University Herbarium (MUHW). Additionally, 10,000 records were obtained from the West Virginia University Herbarium (WVA), and 5,000 from the West Virginia Wesleyan College (WWV) online database (George

B Rossbach Herbarium 2022). Only specimens collected between the years 1959 and 2019 were considered, as we wanted to assess the current distribution of Rosids species, while also having a large enough collection pool to infer spatial pattern. Occurrences were manually filtered for duplicates and erroneous records, such as records that fell outside of the selected time frame or that were not collected in West Virginia. The Taxonomic Resolution Name Service was used to resolve synonymy (Boyle et al. 2013). The occurrences were further filtered with the list of native Rosid species obtained from Weakly (2022), removing non-native taxa. After filtering, approximately 12,700 occurrences remained for subsequent analyses.

Only 300 of the 12,700 occurrences had georeferenced x, y coordinates associated with the collection, the rest only had associated locality data recorded by the collector, such as a locality description string or a nearby municipality. The unreferenced 12,400 occurrences were georeferenced using the batch georeferencing function of the GeoLocate web application (Rios and Bart 2010) which generates a .csv file containing x, y coordinates, along with error polygons and the precision for each georeferenced occurrence. Records that were unable to be georeferenced by GeoLocate (ca. 100) were manually georeferenced by similar localities in the client. Georeferenced occurrences were imported into ArcGIS Pro and mapped, with the default GeoLocate geographic coordinate system, WGS 1984 (ArcGIS Pro, Version 2.9). Level IV EPA ecoregions were overlaid on the map containing the 12,700 Rosid occurrences. Since the Level IV ecoregions have different sizes, we subdivided each ecoregion into 20 km² to calculate the biodiversity metrics. This was done to reduce bias in the biodiversity metrics estimates due to the size of the ecoregions. Grid cells at the border of the state are smaller than the grid cells within the state, as the grid was clipped to fit within the spatial extent of West Virginia.

All biodiversity analyses were performed using R Statistical Software (v4.0.2; R Core Team 2020). Two phylogenetic diversity measures were calculated to assess patterns of spatial diversity of Rosids species, Phylogenetic diversity (PD) and mean pairwise distance (MPD). PD is calculated as the sum of branch lengths for all taxa in an area of interest, while MPD is the mean branch lengths between all pairs of taxa (Faith 1992; Webb et al. 2002). Both metrics were calculated using the Picante package in R (Kembell et al. 2010). Binary presence-absence matrices based upon the 12,700 herbarium records were created for each 20 km² grid square using the ‘pd’ function in Picante. MPD was calculated using the ‘mpd’ function by inputting the presence-absence matrix and an interspecific distance matrix calculated with the ‘cophenetic’ function. Both PD and MPD were used to capture biodiversity information on the Eurosids clade (true Rosids), as these are two biodiversity metrics that measure two distinct aspects of community phylogeny. PD is a proxy for richness that considers the abundance of branch lengths, while MPD considers the range of evolutionary history (Faith 1992; Webb et al. 2002).

Analysis of Variance (ANOVA) was used to determine significant differences in PD and MPD between the 15 Level IV ecoregions using the ‘aov’ function in the base R stats package. For this, the PD and MPD values obtained per grid cell within each ecoregion were added to calculate the total values of PD and MPD per ecoregion. Tukey HSD tests were run to determine which ecoregions were significantly different from each other, with respect to PD and MPD (R Core Team 2020). A null model was developed for both MPD and PD by using the ‘ses.mpd’ and ‘ses.pd’ functions in Picante, respectively. These functions randomize the species present in each grid, calculate the PD and MPD, and then compare the actual PD and MPD obtained with the real species to these randomizations. One thousand randomizations were used, and grid squares were classified as either significantly overdispersed ($p > 0.05$) or significantly clustered

($p < 0.95$) based upon departure from the null value for each grid square. Overdispersion indicates an area with taxa that are not closely related while clustering indicates an area with more closely related taxa.

RESULTS

Grid squares with the highest PD were located in the eastern portion of the state, specifically in pockets in the northeast and southeast (Fig. 8). This coincided with the Forested Hills and Mountains Level IV Ecoregion and lands managed at the federal level, such as the Monongahela National Forest and the New River Gorge National Park. The lowest PD was observed in grid squares in the southwestern portion of the state, coinciding with the Dissected Appalachian Plateau.

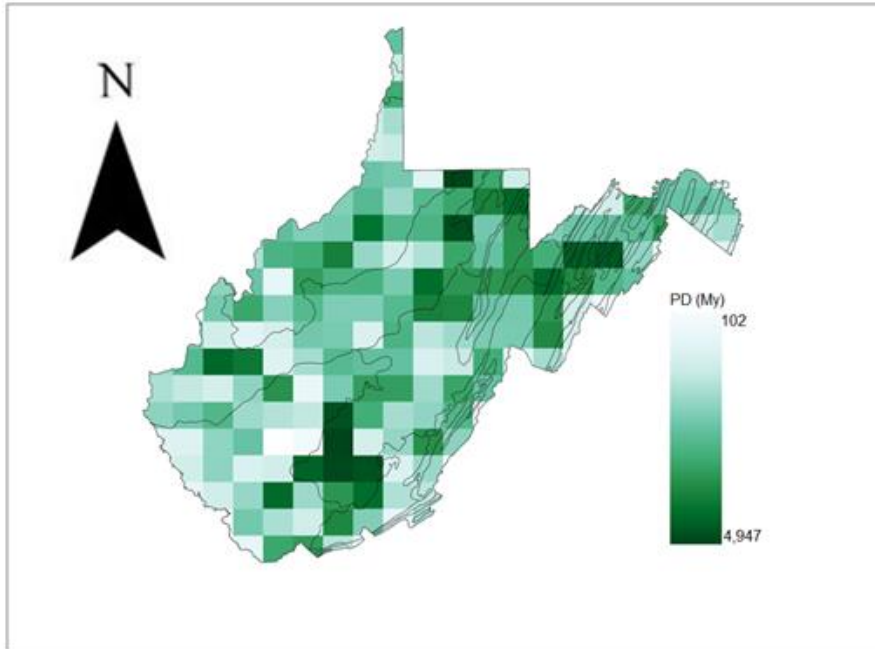


Figure 8. Phylogenetic diversity of Rosids across West Virginia

PD across the Level IV Ecoregions of West Virginia by 20 km² grid squares. Darker green represent higher PD, while white to lighter green represent lower PD. PD is a sum of all branch lengths (in My) for taxa represented in a particular area of interest.

Variation in MPD appeared to be less pronounced across the state (Fig. 9). In terms of breadth of evolutionary time, 180-140 million years, MPD was relatively uniform with few grid squares in the lower range of this evolutionary time. Grid squares with the lowest MPD did appear to occur mainly within the Dissected Appalachian Plateau. The grid squares with the highest MPD occurred along the border regions of the state, which coincide with the Permian Hills in central west and the Greenbrier Karst in the central east.

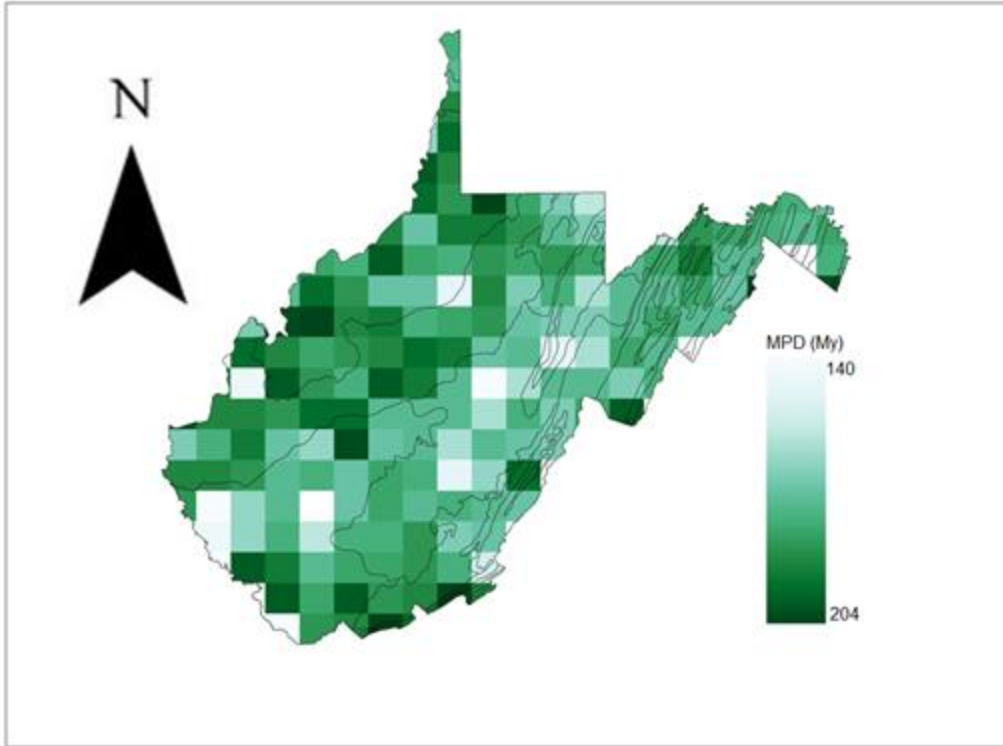


Figure 9. Mean phylogenetic distance of Rosids across West Virginia

MPD across the Level IV Ecoregions of West Virginia by 20 km² grid squares. Darker green represent higher MPD, while white to lighter green represent lower MPD. MPD is a measure of the mean evolutionary distance (in My) of between pairs of taxa in a particular area of interest.

Analysis of the range of PD across all grid squares for each Level IV ecoregion revealed a higher PD in grid squares in the Forested Hills and Mountains Ecoregion (Fig. 10). This ecoregion also had the widest range of PD. While the Forested Hills and Mountains Ecoregion had the highest PD, particularly in areas such as Dolly Sods and New River Gorge National Park, some of the lowest PD observed in this study was in grid squares marked by the highest elevation in the state. While the lowest average PD across all grid cells in an ecoregion was

observed in the Northern Limestone/Dolomite Valleys ecoregion, the grid squares with the lowest overall PD was observed in the Dissected Appalachian Plateau ecoregion of the southwestern portion of the state.

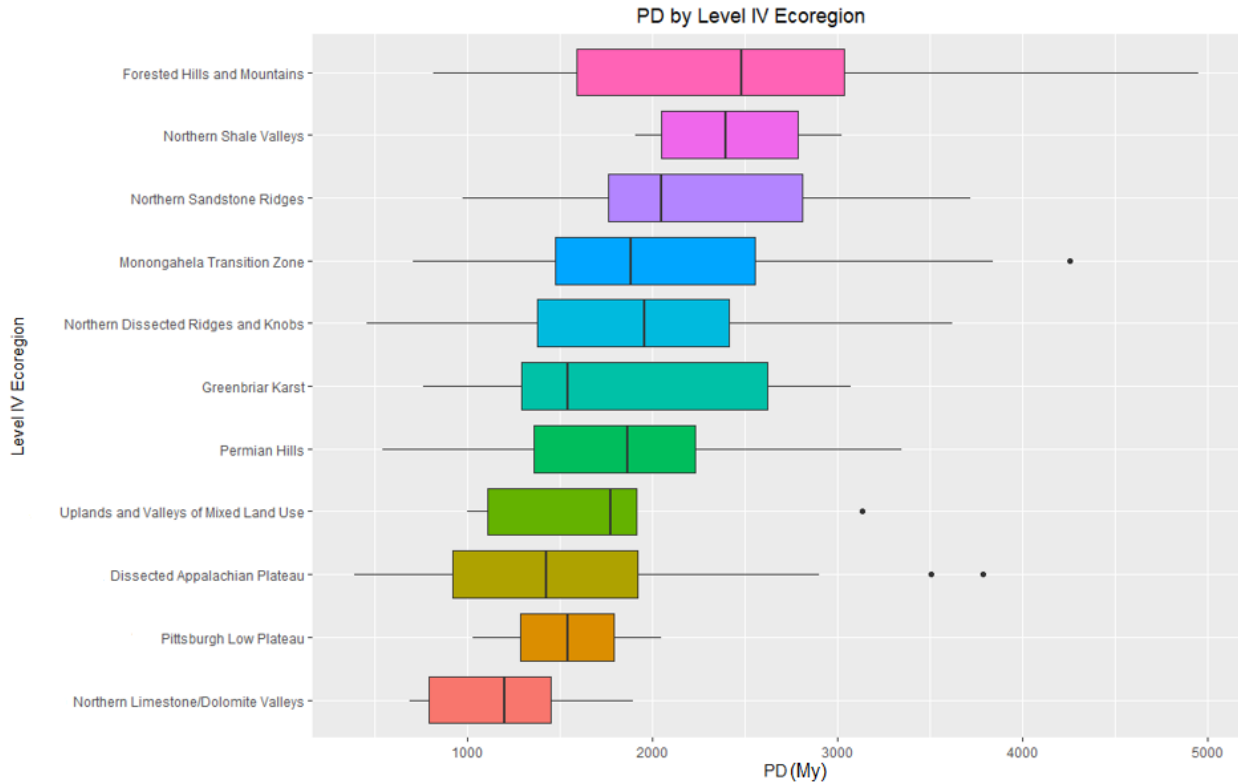


Figure 10. PD by Level IV Ecoregion

Boxplot of PD across all grid squares for each Level IV Ecoregion of West Virginia. Black bars indicate median PD.

Opposite to what was observed across the Level IV ecoregions with PD, median MPD was higher in ecoregions such as Permian Hills and Greenbrier Karst (Fig. 11). Low MPD was observed in the Forested Hills and Mountains ecoregion which had the highest PD. The Forested Hills and Mountains also had a relatively narrow range of MPD when compared with PD for this

ecoregion. The lowest median MPD and grid squares were observed in the Dissected Appalachian Plateau ecoregion, which also had grid squares with the lowest PD. The Dissected Appalachian Plateau had the widest range of MPD, between 183 My and 140 My.

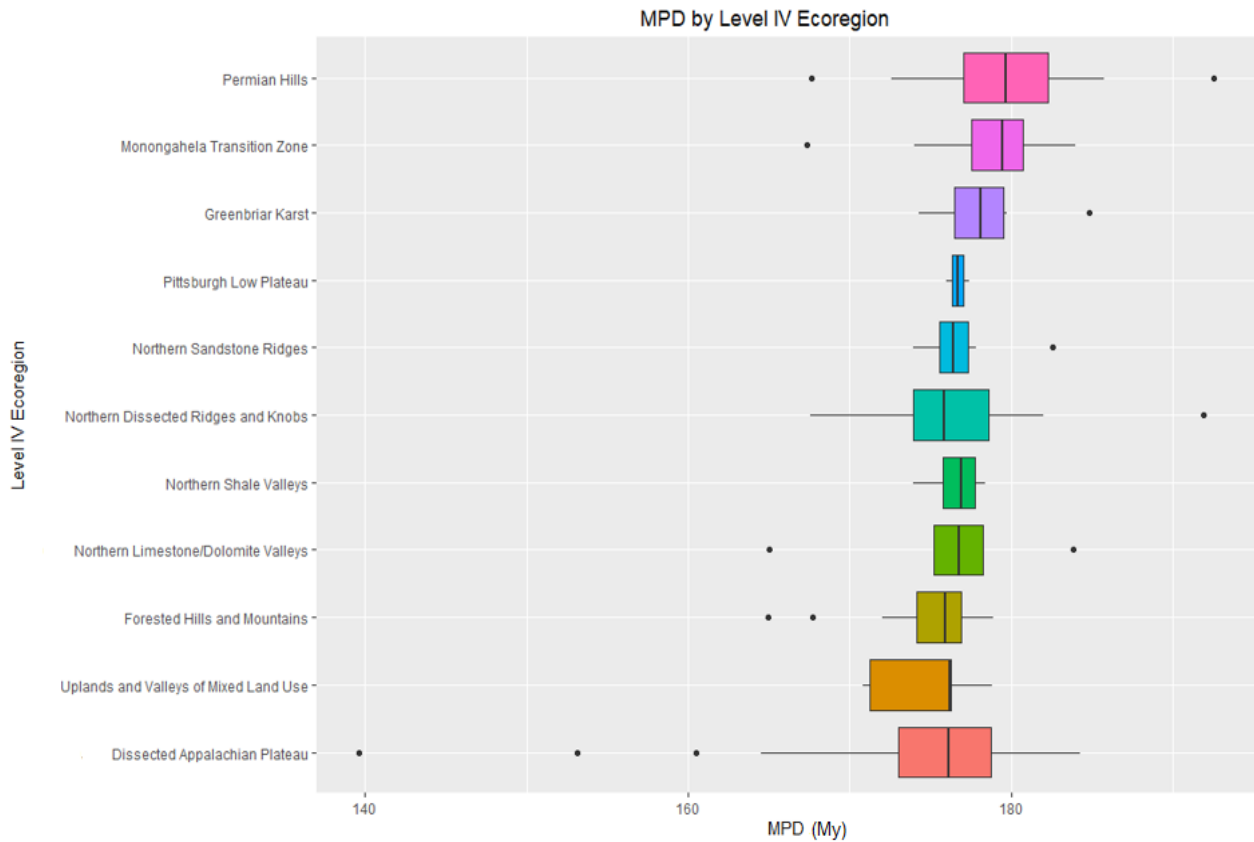


Figure 11. MPD by Level IV Ecoregion

Boxplot of MPD across all grid squares for each Level IV Ecoregion of West Virginia. Black bars indicate median MPD.

ANOVA analyses revealed significant differences between the 15 ecoregions with respect to PD and MPD (Tables 2 and 3). Further analyses with the Tukey HSD test revealed which pairings of ecoregions had significant differences in PD and MPD. Only one pairing,

Permian Hills and Dissected Appalachian Plateau, had a significant difference with respect to MPD ($p < 0.05$). Also, significant difference ($p < 0.05$) was observed in one pairing, Dissected Appalachian Plateau and Forested Hills and Mountains, with respect to PD.

	DF	Sum S	Mean Sq	F value	P value
Level IV Ecoregion	10	727	72.72	2.615	0.00564 **
Residuals	165	4588	27.81		
Significant Codes: 0 '****' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1					

Table 2. One-Way ANOVA comparing effect of ecoregion on PD

One way ANOVA comparing effect of ecoregion on PD ($p < 0.01$). Analysis revealed significant difference in PD over the Level IV Ecoregions.

	DF	Sum S	Mean Sq	F value	P value
Level IV Ecoregion	10	18535158	1853516	2.525	0.00746 **
Residuals	165	121135651	734155		
Significant Codes: 0 '****' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 '' 1					

Table 3. One-Way ANOVA comparing effect of ecoregion on MPD

One way ANOVA comparing effect of ecoregion on MPD ($p < 0.01$). Analysis revealed significant difference in MPD over the Level IV Ecoregions.

The randomization tests revealed areas of significant overdispersion and significant clustering with respect to MPD and PD. Overdispersion indicates areas in which taxa are more distantly related and clustering indicates areas in which taxa are more closely related than expected due to random chance. There were no grid squares with significant clustering, only grid squares with significant overdispersion for PD (Fig. 12). For MPD, grid squares with significant overdispersion were concentrated mainly in the eastern half of the state, while grid squares with significant clustering were concentrated in the western half of the state (Fig. 13). Overall, there were an equal number of significantly overdispersed and significantly clustered grid cells in the model for MPD.

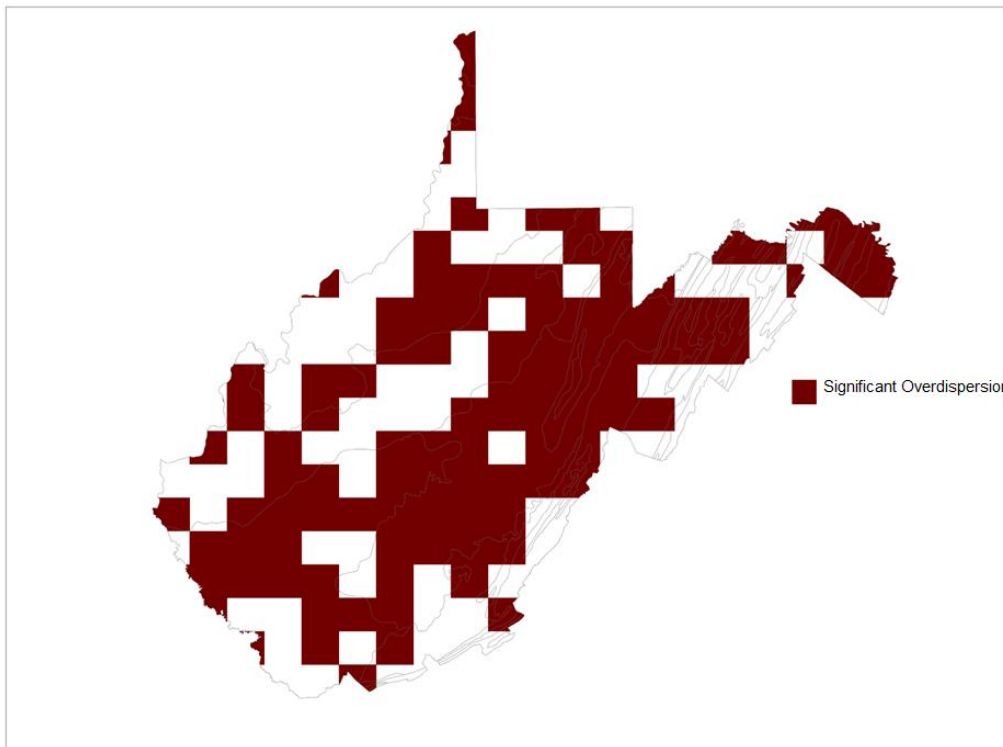


Figure 12. Significant overdispersion of PD

Phylogenetic Overdispersion within each grid square based on comparison to null model where tips are randomly shuffled across the phylogeny. Significant overdispersion represents a significant departure from null model ($p < 0.9$). This indicates grid squares with significant PD.

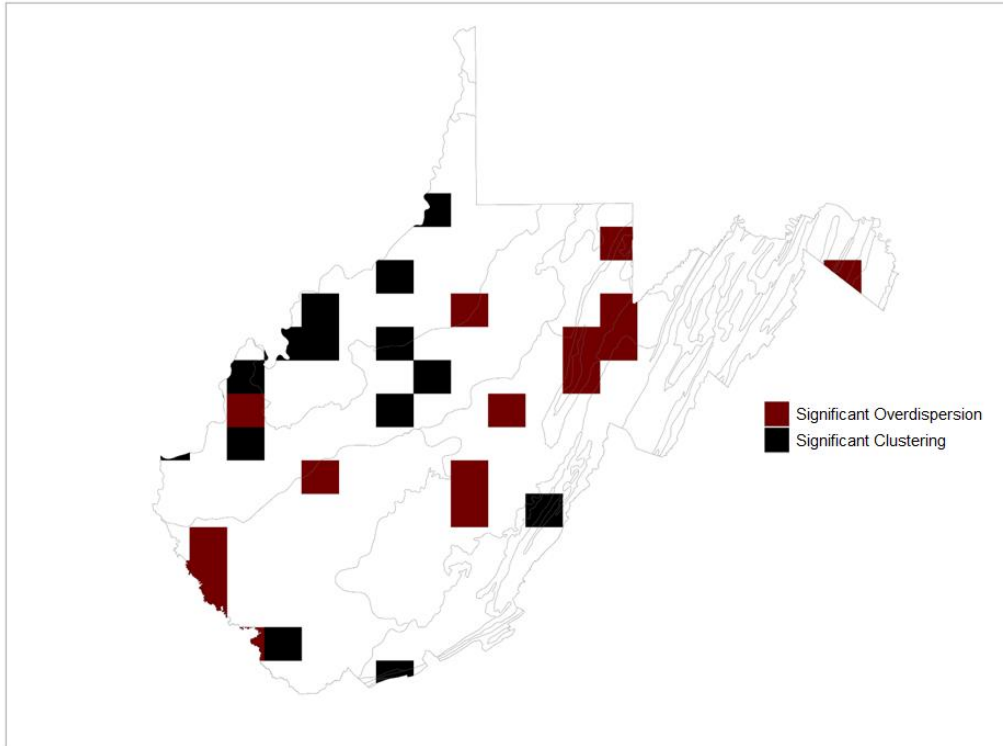


Figure 13. Significant overdispersion and significant clustering of MPD

Phylogenetic Clustering and Phylogenetic Overdispersion within each grid square based on comparison to null model where tips are randomly shuffled across the phylogeny. Significant clustering and significant overdispersion represents a significant departure from null model ($p < 0.05$ and $p > 0.9$ respectively). Significant clustering indicates grid squares in which taxa are significantly more closely related in geologic time, while significant overdispersion indicates grid squares in which taxa are significantly more unrelated in geologic time.

The phylogeny generated from this study revealed patterns of diversity statewide, in terms of the different plant orders (Fig. 14). The order Rosales was the most abundant in terms of number of species and diversification, comprising 92 taxa, about 25% of the total native Rosids species considered. Other abundant and speciose orders included: Malpighiales (72 taxa), Fabales (58 taxa), and Fagales (39 taxa).

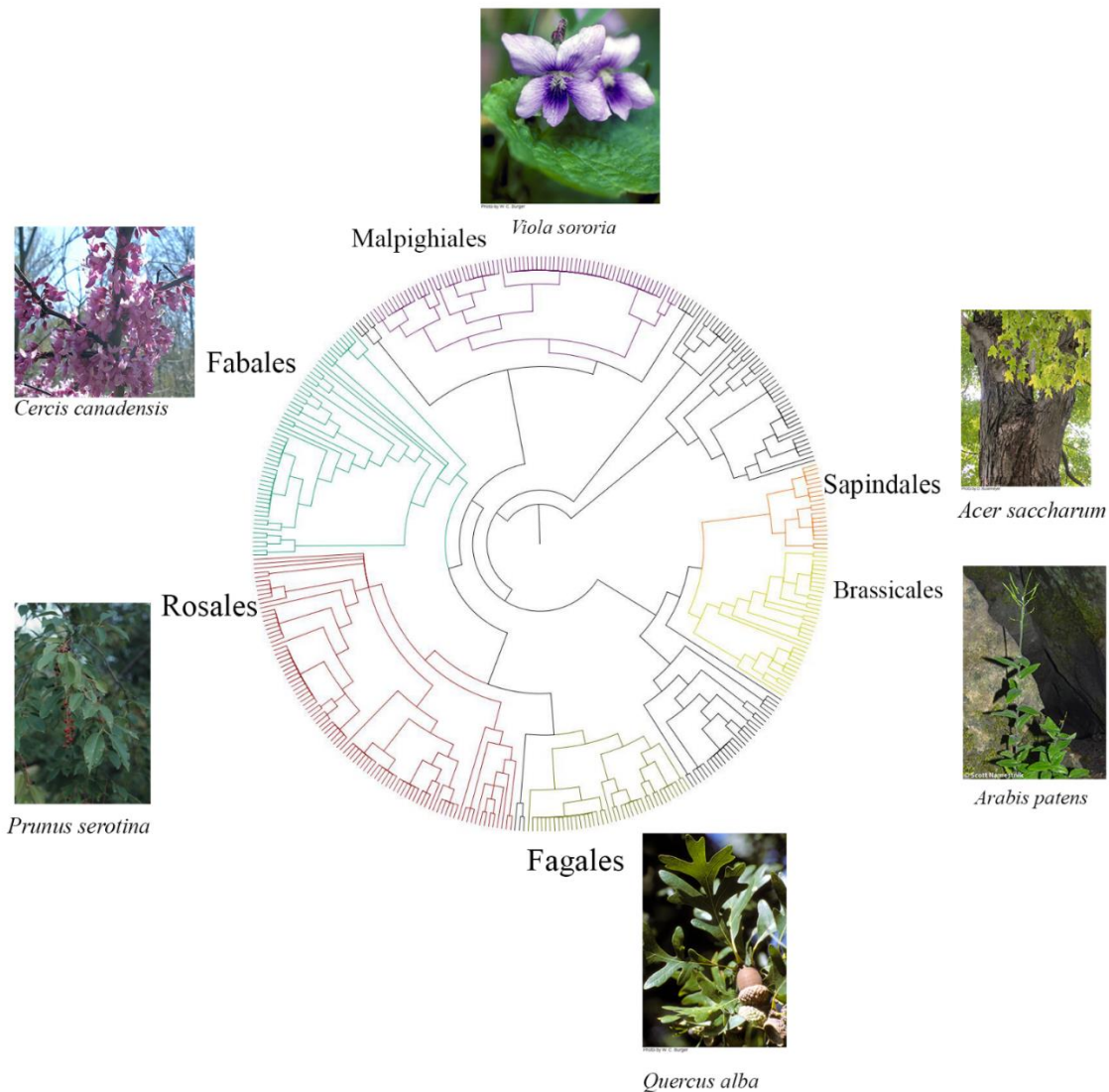


Figure 14. Inferred phylogeny of native West Virginia Rosids

Phylogeny of native West Virginia Rosids inferred using Phylocom (include reference for software here). Mega tree used to parse native species was derived from Slik et al. (2018). Major orders are highlighted, and an example species is included for each order.

DISCUSSION

Spatial Patterns of Phylogenetic Diversity in West Virginia

Our analyses revealed distinct spatial patterns of phylogenetic diversity of the Rosids across West Virginia. Notably, high phylogenetic diversity was observed in the Forested Hills and Mountains Ecoregion of the northeastern portion of the state, specifically the Monongahela National Forest and New River Gorge National Park. The Monongahela National Forest has been a federally protected area since 1920. Subsequent management plans after clear cutting harvesting ceased in the 1980s promoted restoration of these forests to spruce and oak (Van Gundy and Morin 2021). This might be what the high PD levels are reflecting. The lowest PD was observed in the Dissected Appalachian Plateau, in the southwestern portion of the state. Low diversity in this ecoregion may be a consequence of land use practices such as logging and mining. The effects of mountain top removal mining, which occurs in the Dissected Appalachian Plateau, has been well studied and has been linked to the decline of biodiversity in central Appalachia (Wickham et al. 2007). There was significant difference in PD by ecoregion, but the only two individual ecoregions that were significantly different from one other were the Dissected Appalachian Plateau and the Forested Hills and Mountains. Prior research has shown a link between differences in PD and ecoregion (Spalink et al. 2018a; Allen et al. 2019). Ecoregions may have environmental filters like climate, elevation, and others that constrain the plant assemblages present. For example, the Northern Limestone/Dolomite Valleys, which had some of the lowest PD may have more environmental filters that constrain PD, than the Forested

Hills and Mountains ecoregion. Previous studies have investigated this, with indications that environmental conditions, such as climate, substrate, and elevation, may limit richness in species composition (Qian et al. 2015).

Analyses of mean pairwise distance show that the ecoregion with the highest MPD, or breadth of evolutionary history, is the Permian Hills ecoregion. This area is characterized by the Ohio River watershed and rugged terrain that has made farming difficult and made this area less likely to experience change in land cover due to human use (Woods et al. 1999). It was surprising that this area would have the highest MPD but one of the lowest PD. This would mean that while this ecoregion is less diverse in the number of distinct evolutionary histories, it is more diverse in terms of the breadth of evolutionary history for the assemblage as a whole. A possible explanation for this, is that these ecoregions contain more rare lineages, which would increase MPD, but not necessarily PD. On the other hand, the ecoregion with the lowest MPD was the Dissected Appalachian Plateau, which also had the lowest PD. This suggests that this ecoregion has both less richness and divergence with respect to its assemblages, a possible consequence of the degradation of this habitat due to logging and mountain top removal coal mining activities (Wickham et al. 2007; Palmer et al. 2010; Maxwell et al. 2012). Studies in other areas/states have found similar patterns of low diversity measures correlated with human activities. For example, a study by Van Meerbeek et al. (2014) found increasingly low measures of PD in agricultural lands of higher intensity, while Turner et al. (2002) showed a decline in area covered by species diverse oak-pine forests in areas disturbed by human activity like mining and logging.

When comparing significant differences between each individual ecoregion in MPD, we observed a significant difference between the Permian Hills and Dissected Appalachian Plateau and between the Monongahela Transition Zone and Dissected Appalachian Plateau. The Permian

Hills and Monongahela Transition Zone ecoregions had the highest MPD, while the lowest MPD was observed in the Dissected Appalachian Plateau. While the highest diversity does exist in mainly managed lands, high diversity also exists in areas that are outside of these managed areas, such as the Permian Hills which had the highest MPD. It may be prudent to target these areas for conservation priorities, as they may be threatened by the effects of human land use and climate change. Also, it will be advisable to continue conservation actions in areas already under federal and state management, and to monitor these areas as the effects of climate change impact local plant assemblages (Feeley and Silman 2011; Saladin et al. 2016).

Identifying areas of significant overdispersion and significant clustering is important in delineating specific regions of exceptionally high or low diversity. In terms of exceptionally high diversity, these areas may contain rare taxa, either in abundance or range. For example, the rare species *Euphorbia purpurea* Fernald (Darlington's Glade Spurge) occurrences were found in the eastern portion of the state, which contained the grid squares with the highest diversity. Our analyses revealed that assemblages in the eastern portion of the state, in ecoregions such as the Forested Hills and Mountains and Upland Mixed Use Valley had more grid squares of significant overdispersion. This may be due to heterogeneity of these ecoregions, with differing habitat types that contain unique assemblages (Van Gundy and Morin 2021). In terms of PD, there was only significant overdispersion observed across the state. There appeared to be more grid squares of significant overdispersion in the eastern portion of the state, which was consistent with the observed PD results.

For MPD, the grid squares with significant overdispersion were concentrated mainly in the eastern portion of the state, while the grid squares with significant clustering were concentrated in the western part. As with the PD results, this indicates that the assemblages of

the eastern portion of the state are more distantly related than assemblages in the western part of West Virginia. This may be reflective of land management initiatives at the federal and state level in the eastern portion of the state.

Study Specific Phylogeny

Our study specific phylogeny shows a predominance of the Rosales order, which included 92 of the 361 Eurosoid species (core Rosid group). This order includes trees and shrubs that are important to the composition of West Virginia forests such as cherries (*Prunus* L.), hawthorns (*Crataegus* Tourn.), and elms (*Ulmus* L.). This relative proportion of taxonomic groups was consistent with prior literature as was the evolutionary relationships recovered (Wang et al. 2009; Sun et al. 2020). These prior studies recovered a dominance of the Rosales order, which we found in this study. The second most diverse order was Malpighiales, which contains important families such as Violaceae (violet family), Euphorbiaceae (spurges), Salicaceae (willows), Passifloraceae (passion flowers), among others. Genus *Viola* L. for example, contains many important forbs that provide food for pollinators and some species have seed dispersal interactions with ants (Beattie and Culver 1981; Weakley 2022). The third most diverse order in our phylogeny was the nitrogen fixing order Fabales, which contains families like Fabaceae (legume family) and Polygalaceae (milkworts). Taxa represented in this order include members of genera *Desmodium* Desv. (tick trefoil) and *Lespedeza* Michx. (bush clover). Both genera are important for nitrogen fixation as they make nitrogen available to other associated plants (Sprent et al. 2017).

We used a pre-existing phylogenetic tree to which we parsed our list of species. A prior study tested the differences between using Phylomatic to infer a phylogeny and a phylogeny inferred from specific genes (Allen et al. 2019). They found minimal differences in spatial

biodiversity between both. It should be noted however, that the inferred phylogeny is dependent upon the mega tree used to parse the study specific taxa, and that branch length calibration will be dependent upon the method used. For our study, we inferred a chronogram with an ‘ages’ file containing estimated ages of various groups of angiosperms published by Wikström et al. (2001). This file also happens to be the default ‘ages’ file included with Phylocom. Wikström et al. (2001) phylogeny had good support values and has been used in calibration of chronograms in prior studies (Johnson et al. 2015; Chai et al. 2016).

Limiting Factors and Future Directions

Herbaria data provides a broad sampling of specimens across geographic and temporal ranges, however biases related to the collection process do occur. Collections are usually made in areas within a short distance of herbaria, from national and state parks, near roads, etc., whereas remote areas or areas of difficult access are often under-collected. Also, as plant collectors tend to specialize in particular groups, collection biases may occur in the taxa collected, with certain taxa being underrepresented in herbaria catalogs. These forms of collection biases are well known and must be accounted for when conducting herbaria informatic analyses (Loiselle et al. 2008; Daru et al. 2017). Grid squares of high diversity near the West Virginia University and the Marshall University campuses may indicate collection bias in our study. Potential collection biases may be accounted for by utilizing species distribution modeling software such as maxent (Phillips et al. 2006).

Further analyses may be done on other major angiosperm clades in West Virginia or more broadly on the entire assemblage of angiosperms within this state. Distribution modeling

software, in conjunction with climate change models, may be used to identify specific areas that may potentially be vulnerable to the effects of climate change. Also, analyses may be done with consideration of invasive species and how their spread has modified diversity. The importance of invasive species is expected to increase under changing climate regimes, so understanding where and how invasive species spread will be important for conservation efforts (Corlett 2016).

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APPENDICES

APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER



Office of Research Integrity

November 30, 2020

Zachary Scott Shamblin
2820 4th Avenue
Huntington, WV 25702

Dear Mr. Shamblin:

This letter is in response to the submitted thesis abstract entitled "*Phylogenetic Diversity of the Rosids Clade in West Virginia Using Herbaria Data.*" After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

A handwritten signature in blue ink that reads 'Bruce F. Day'.

Bruce F. Day, ThD, CIP
Director

**APPENDIX B. SPECIES, VOUCHER, COUNTY, STATE, COUNTRY. MUHW:
MARSHALL UNIVERSITY HERBARIUM.**

Cortinarius sp.—MUHW No. 699: Wayne County, West Virginia, US.

Dicranum fulvum Hook.—MUHW 3045: Tucker County, West Virginia, US.

Cladonia furcata (Huds.) Schrad.—MUHW No. 123: Wayne County, West Virginia, US.

Pinus virginiana Mill.—MUHW No. 9: Putnam County, West Virginia, US.

Banksia sp.—MUHW Teaching collection: Provenance unknown.

**APPENDIX C. LIST OF ITEMS PURCHASED FOR PHOTOGRAPHING 3-D
SPECIMENS IN THE PRESENT STUDY.**

Amazon Basics 50-inch Lightweight Camera Mount Tripod Stand With Bag

https://www.amazon.com/gp/product/B00XI87KV8/ref=ppx_yo_dt_b_asin_title_o03_s00?ie=UTF8&psc=1

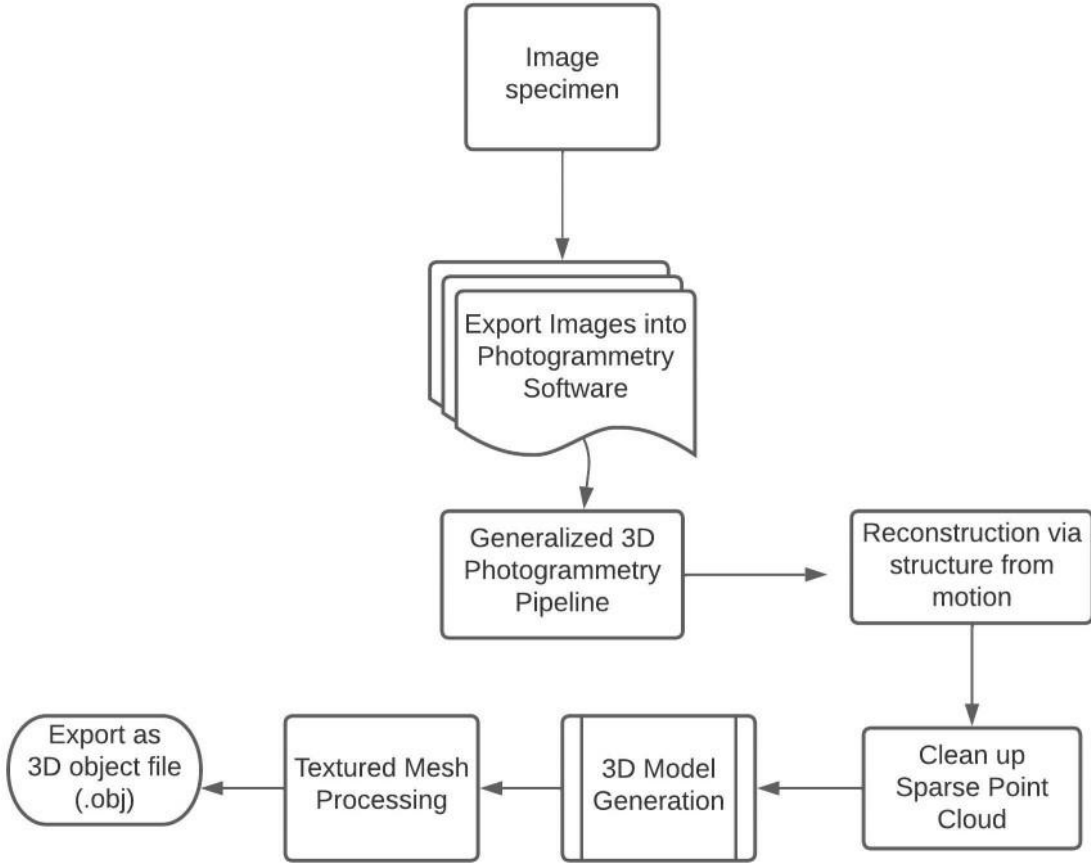
Kootek 11 Inch Rotating Cake Turntable, Turns Smoothly Revolving Cake Stand Cake
Decorating Kit Display Stand Baking Tools Accessories Supplies for Cookies Cupcake (White)

https://www.amazon.com/dp/B082PZ5C8W?ref=ppx_pop_mob_ap_share

Artliving Two Part Adjustable Clear Acrylic Plastic Display Stand Easel (Set of 6)

https://www.amazon.com/dp/B06XSJ2VCN?ref=ppx_pop_mob_ap_share&th=1

**APPENDIX D. WORKFLOW FOR IMAGING SPECIMENS AND PROCESSING
TEXTURED MESH.**




APPENDIX E. PROTOCOL FOR PRODUCING A 3D MODEL USING THE 3DF ZEPHYR FREE SOFTWARE.

Sparse Point Cloud Generation

1. Create a new workflow by opening a “New Project” under the Workflow tab in the top ribbon.
2. Import images either by dragging and dropping them into the workspace or by importing from the folder in which the images are located. [Note: 3DF Zephyr Free does not allow more than 50 images to be incorporated into a mesh. If you have more, it will be necessary to remove images.]
3. On the Camera calibration page, click “Next” (the software will automatically import camera metadata from the image files).
4. Set “Category” for generation of sparse point cloud as “Surface Scan”, leave “Presets” as default. [Note: If multiple images fail to be incorporated into the point cloud, then the “Presets” on the Camera Orientation presets page may be switched to “Deep.” This will allow for more image matches but will also result in a slower point cloud generation.]

Editing 1 (optional)

To remove the background from your images (i.e. rotating tray, stand, etc.), use the built-in masking tool before creating a new workflow (Step 1) by clicking the Masquerade icon  in the ribbon, on the top of the window.

Densification, 3D Model Creation, and Textured Mesh Processing

5. Generate the 3D model using the sparse point cloud produced in the previous step. Open “3D Model Creation” under the Workflow tab. [Note: The 3D Model Creation wizard

performs two tasks in succession, the first being the creation of a dense point cloud and then the creation of an untextured 3D model. It is suggested that the default settings are used for both of these steps.]

6. Create the textured mesh by opening “Textured Mesh Generation” under the Workflow tab.
7. Export the final textured mesh as a 3D object (.obj) file by clicking “Export Textured Mesh” underneath the Export tab in the top ribbon. This will export a folder containing three files, .obj, .mtl, and .jpg. These three files are necessary to open a colored 3D object. The .obj file can be opened in any Windows computer that has a 3D Viewer application.

Editing 2 (optional)

After doing the masking (Editing 1), there may still be some background remaining. This can be further cleaned after the textured mesh generation (Step 6) with the Editing tab at the right-hand side of the project window. Unwanted points may be deleted by hand or selected for and deleted according to undesired attributes. For example, the color of the background can be selected and all points containing this color will be deleted. It is advised however, to use this cautiously as this can generate holes in the model.