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A Method for Citizen Scientists to Catalogue Worldwide *Chlorociboria* spp. Distribution

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Abstract: The blue-green pigment known as xylindein that is produced by species in the *Chlorociboria* genus is under heavy investigation for its potential in textile dyes, wood dyes, and solar cells. Xylindein has not yet been synthesized, and while its production can be stimulated under laboratory conditions, it is also plentiful in downed, decayed wood in forested lands. Unfortunately, little is known about the wood preference and forest type preference for this genus, especially outside New Zealand. To map the genus would be a massive undertaking, and herein a method by which citizen scientists could contribute to the distribution map of *Chlorociboria* species is proposed. The initial trial of this method found untrained participants successfully identified *Chlorociboria* stained wood in each instance, regardless of forest type. This simple, easy identification and classification system should be well received by citizen-scientists and is the first step towards a global understanding of how xylindein production might be managed for across various ecosystems.

Keywords: *Chlorociboria*; spalting; xylindein; citizen-scientist; sampling protocol

1. Introduction

Large-scale organism sampling studies that focus on the species level are often costly and difficult to achieve if done by a single scientist team [1,2]. Relying on citizen scientists to expand and contribute to field data on different research topics, such as ornithology [1], mycology [3], and entomology [4], is increasingly common. Initiatives such as eBird, developed by Cornell University, has shown the feasibility, accuracy, and reliability of data generated by citizen scientists [4,5].

Citizen scientist initiatives focus on citizen groups that are in contact and have a degree of knowledge about the subjects of study. eBird, for example, collects data for bird identification and distribution, and is targeted at bird watching hobbyists around the globe [6]. Another example of reliance on citizen-scientists for data collection is the survey of fungi in Warwickshire in the United Kingdom, where local members of various mycological societies were trained by experts, and, for fifteen years, collected one of the most detailed and reliable data sets of the fungi in the area [7]. Another example is the ten-year chanterelle (*Cantharellus cibarius*) project, which evaluated different mushroom harvesting techniques in ten plots and how it affected future fruiting of the species [8–10]. A third example, specific to mycology in the North American Pacific Northwest (PNW), used amateur mycologists belonging to mushroom societies of the PNW, such as the Oregon Mycological Society, for one of the first long-term studies about the impact of different harvesting techniques on wild mushrooms [8–10]. Most of the members in these societies are college educated and had a high degree of mycological knowledge [11].

Reliance on citizen scientist data can make big sampling studies more affordable, and also makes science more approachable to the general public. By involving citizens in scientific studies, a wider network is made, where the scientific team and the population are more involved with the objective of achieving a shared goal [2].

The genus *Chlorociboria* (phylum Ascomycota) produces wood spalting (pigmentation of wood produced by fungi) [12–15]. This genus is present in Europe [15], North America [16–18], New Zealand [19], India [20] and certain areas of Central and South America [21–23]. Species of this genus are found growing on decaying wood often already colonized by white rot fungi, with species having been isolated from both hardwoods and softwoods [18,19,24,25]. There are two notable species found in North America and Europe, *Chlorociboria aeruginosa* (Oeder) Seaver ex C.S. Ramamurthi, Korf and L.R. Batra and *Chlorociboria aeruginascens* (Nyl.) Kanouse ex C.S. Ramamurthi, Korf and L.R. Batra (1, 3–4, 6–7) [16–18,26,27]. Like many in this genus, these two species create a blue-green color in wood due to the production of the pigment xylindein [28,29], and have been historically used in European woodworking as early as the 13th century [30–33].

Databases containing the distribution of *Chlorociboria* spp. are also available from mycological societies, such as the Danish Mycological Society and in online resources as the Global Biodiversity Information Facility (GBIF) [34]. However, these databases carry information that is usually focused in certain areas (such as the mycological societies), or they rely on the information collected by other institutions that focus on other areas of interest. This focus on one or two particular genera or types of fungi generates geographical gaps that can benefit from citizen-scientists' data collection.

Currently, there is renewed interest in xylindein for use in UV-resistant wood stains, textile dyes [35–37], and solar cells [38]. Attempts to synthesize xylindein have been unsuccessful [39]; however, pigment production of *Chlorociboria* can be stimulated under certain laboratory conditions [40,41]. The pigment can also be extracted from the naturally occurring fungal substrate using non-polar solvents, with dichloromethane (DCM) the most effective [42]. Experiments have also been done using centrifugal partition chromatography to isolate and purify xylindein [43], and the extracted pigment has been used directly on wood [35,36], bamboo [44] and textiles [45,46] to test its effectiveness at causing coloration to mimic the effects of *Chlorociboria* natural growth.

With the increasing research interest in xylindein and the ability to use the naturally occurring pigment in addition to generating xylindein in the lab, distribution and density of *Chlorociboria* species are of interest. Particularly valuable would be the management of woodland understories (especially the slash wood) for *Chlorociboria* by woodland owners. This would require a better understanding of wood species preference and habitat preference for the genus than we currently have. In addition, understanding wood preference of *Chlorociboria* species would help with laboratory production of xylindein, as *Chlorociboria* species are notoriously difficult to grow in the lab, and require specialized wood-amended media [40] for maximum xylindein production.

The purpose of this work was to develop an accessible and repeatable method for citizen scientists to map occurrences of the *Chlorociboria* genus across the world, in an effort to catalogue its distribution, habitat preference, and wood species preference, with a mind to future biotechnology developments.

2. Materials and Methods

2.1. Sampling Area—USA

Samples of blue-green wood were collected in the Benton County area of Oregon, USA, where sightings of blue-green stained wood had been previously reported. Two forests were selected based on their accessibility and recreational value. The first forest was Mary's Peak, which belongs to the Siuslaw National Forest. On this forest, Dinner Creek and Woods Creek were sampled. The second forest was the McDonald-Dunn forest, which belongs to the College of Forestry of Oregon State University. There, three creeks were sampled: Soap Creek, Jackson Creek, and Oak Creek.

The collection was focused on riparian areas due to the fact that previous reports on *Chlorociboria* species state their preference for humid areas [18]. The main factors for the selection of areas to be sampled were: drivability, accessibility and terrain. The sampled creeks were close to delimited paths. USA samples were collected from November 2015 to March 2016.

2.2. Surveying Area—Peru

Peruvian surveying areas were within the Inkaterra property, located in the district of Las Piedras, in Madre de Dios, Peru. This surveying area was selected due to the reported biodiversity of flora and fauna [47,48] and its accessibility. Previous scouting and collections by experts in this area discussed an abundance of spalting fungi [49].

In this area, two sites were sampled. The first one was coded as Reserva Amazonica (RA). Reserva Amazonica (RA) corresponds to a lower terrace tropical rainforest and has a perennial wetland within the area [50]. The forest at RA corresponded to a primary forest with little disturbance, besides the use of the area for ecotourism. The other site was coded Hacienda Concepcion (HC). This site corresponded to a high terrace [50] and the forest corresponded to secondary growth mixed with cocoa (*Theobroma cacao*). Surveying (no samples were collected due to permitting restrictions) was done in June of 2016.

2.3. Training

Field crews, consisting of three to six non-specialized people, were trained on the rapid assessment of *Chlorociboria* spp. on downed wood by direct observation of sampling techniques (described below). The training consisted of recognizing the blue-green coloration of the inner downed wood, which is characteristic for this genus, and the recognition of the fruiting bodies produced by this fungus, locally known as "elf's cup". The field crews went into the selected areas, first supervised by a graduate student or an instructor and then alone or accompanied by a field guide (Field Station Inkaterra only).

2.4. Sampling

Samples were collected during the rainy seasons of the respective countries, which increased the likelihood of encountering *Chlorociboria* spp. fruiting bodies. Sampling was done in two different manners, differing due to safety concerns in the Amazon. For the USA, all sampling was done using a line-intercept method along 400 m transects on both sides of the stream. Transects were run parallel to the stream, 8 m from the edge of the creek. A Spencer® 950 loggers tape (Spencer Products CO., Seattle, WA, USA) was used to determine distance from the stream, being re-measured approximately every 15 m to account for bends in the stream. All downed wood (trees and branches) with a diameter of at least 5 cm encountered along the 400 m transects were sampled, using a ruler to determine whether the wood met this requirement. The starting location of the transects was determined by choosing the closest point of the stream from the entry site using Google Maps (version 4.28.0, Google, Mountain View, CA, USA) for iPhone as a reference. The entry site was defined as the closest area to the creeks accessible by car.

For Peruvian surveying, transects were confined to known, premade trails in the area, and researchers were allowed to venture only up to 15 m perpendicular to them due to safety concerns. The starting point was defined as the area closest to the field station or lodge.

Regardless of country, all downed, dead wood within 8 m of the trail/transect (no wood smaller than 5 cm was considered) was opened using a machete to determine if *Chlorociboria* spp. was present. The wood was cut 3 cm deep along a 10-cm segment on the top side of the log, with repeat cuts every 5 cm along the length of the log. This method was selected because spalting fungi mostly do not produce fruiting bodies, and it is therefore necessary to look internally into the dead logs to find colonization evidence (the blue-green color, which is unique to the genus [15,21,30,42,51–53]).

The presence of fruiting bodies was also used when fruiting bodies occurred. Only downed, dead wood was sampled, as *Chlorociboria* spp. is generally a slow growing, wood decomposing fungus [54]. If no pigmentation was found after going approximately 3 cm deep into the wood, it was considered to not contain *Chlorociboria* spp. If pigmentation was present, the GPS location was determined using the Easy GPS application for iPhone (Apple, Cupertino, CA, USA) made by Kraus und Karnath GbR 2kit (Düsseldorf, Germany) for the forests located in Benton County, while a Garmin GPSmap 64s (Garmin, Schaffhausen, Switzerland) was used in the Madre de Dios, Peru area, as phone signal was limited.

Sample collection was performed in the US site only due to permitting restrictions in Peru. The samples were from downed wood that had the characteristic blue-green pigmentation and that were not completely decomposed (samples which did not crumble in the collector's hand), as some structure integrity was required for further examination. The size of the samples collected was a minimum of 5 cm in width and length, and at least 0.4 cm in thickness.

The GPS coordinates were then input into an Excel spread sheet (Microsoft) and uploaded to ArcMap (ESRI, version 10.5.1, Manufacturer, Redlands, CA, USA) to generate maps of the collection sites.

2.5. Wood Identification

Researchers were not allowed to remove wood from Peruvian forests; therefore, only field wood ID could be performed. For US occurrences, any samples that occurred on wood hard enough to be collected were taken to the Applied Mycology laboratory at Oregon State University to determine the wood species. Wood slides of 16 µm were obtained utilizing a Spencer-Buffalo Company microtome to create uniform sections of each plane. The obtained wood slides were placed on a glass slide (VWR®, Radnor, PA, USA) and covered with a #1.5 glass cover-slip (VWR®). The slides were then examined using a Nikon Eclipse Ni-U light microscope (Nikon Instruments Inc., Melville, NY, USA) and photographed using a Nikon DS-Ri2 microscope camera.

Once the images of the wood slides were obtained, different anatomical aspects were noted. The first aspect was to identify if the wood was a conifer or a hardwood species by analyzing the presence of vessels. Ray types (upright and/or procumbent), length and width (unicellular and/or multicellular) were also annotated. The parenchyma type was described per specimen and special characteristics (as presence of gums or prismatic crystals) were noted. After collecting the information per wood sample, the characteristics were run through the North Carolina State University Insidewood data base [55].

2.6. Verification

Microscopic verification of the wood species, as well as confirmation of *Chlorociboria* species colonization, was done by wood anatomy experts using a Nikon Eclipse Ni-U microscope to evaluate the presence of hyphae and the location of the pigment in the wood.

For the Peruvian samples, the confirmation of the possible presence of *Chlorociboria* spp. was performed by visual inspection by a professor and/or postdoctoral researcher, and was entirely based upon the correct color being present in the wood (blue-green). Verification was necessary for the sampling, as this was the first time the method was applied and verification was required to make sure the collections and fungal identification by wood pigmentation was performed correctly, and that the explanations given were sufficient for a non-technical person.

3. Results

3.1. Sampling

In the USA, the trained students successfully identified eight logs that contained *Chlorociboria* along the various transects/trails. All of the logs identified by the students as having *Chlorociboria* species were confirmed as hosts of *Chlorociboria* spp.

In Peru, identification of *Chlorociboria*-stained wood was made through visual determination of the pigment, as no specialized equipment was available for further inspection. However, per the methods for the USA identification, a professor and/or post-doctoral research did verify that the correct color was present to indicate *Chlorociboria* spp. All student identifications were correct.

3.2. Site Accessibility

The selection of the sampling sites allowed for easy accessibility by one person or a team up to three. As accessibility is a main factor for 'citizen-scientists', this was taken into consideration for the experimental design. Using this technique, downed trees and branches carrying *Chlorociboria* spp. were successfully identified with minimal danger to the participants. The use of an iPhone app was successful (and highly accessible), and the consumer grade GPS used for the mapping in Peru also performed well, and all data points were saved (Tables 1 and 2).

Table 1. GPS coordinates of fallen wood containing *Chlorociboria* spp. taken with Easy GPS in Mary's Peak and McDonald-Dunn Forests.

Location	Sample	Latitude (Decimal Degrees)	Longitude (Decimal Degrees)	Wood Species
Oak Creek	O1	44.60565	−123.33231	*Acer macrophyllum*
	O2	44.60805	−123.33216	-
Soap Creek	S1	44.64328	−123.32298	-
	S2	44.64355	−123.32475	*Acer macrophyllum*
	S3	44.64348	−123.32486	-
	S4	44.64348	−123.3233	-
	S5	44.64366	−123.3233	-
Woods Creek	W1	44.54433	−123.45474	*Acer macrophyllum*
	W2	44.54446	−123.45468	*Acer macrophyllum*
	W3	44.54446	−123.45468	*Acer macrophyllum*
	W4	44.54446	−123.45088	-
	W5	44.54446	−123.45048	*Acer macrophyllum*
	W6	44.54448	−123.44968	-
Dinner Creek	D1	44.47248	−123.4909	*Acer macrophyllum*
Jackson Creek	J1	44.6134	−123.29206	*Salix* spp.

Table 2. GPS coordinates of fallen wood with suspected *Chlorociboria* spp. taken with GPSmap 64s in Field Station Inkaterra, Madre de Dios, Peru.

Location	Sample	Latitude (Decimal Degrees)	Longitude (Decimal Degrees)	Wood Species
Reserva Amazonica	331A	−12.5333	−69.0437	-
	441C	−12.5347	−69.0481	-
	578	−12.5385	−69.0505	-
	641	−12.5373	−69.0544	-
	679B	−12.5328	−69.0511	-
	709	−12.5359	−69.0518	-
	737	−12.5293	−69.0455	-
	755B	−12.5282	−69.0451	-
	941B	−12.5258	−69.0456	-
Hacienda Concepcion	1001B	−12.6068	−69.0816	-
	1201	−12.6058	−69.0789	-
	1359	−12.6078	−69.0736	-

3.3. Wood Species Identification

A total of eight wood samples were collected. Of these samples, seven were found to be big leaf maple (*Acer macrophyllum* Pursh), and all of these seven samples were correctly identified to the genus (maple) by the participants without the use of a microscope. The samples presented exclusive procumbent multiseriate ray cells. Helical thickening on the vessel cell walls was also present, and this characteristic determined the identification of the specimens as a big leaf maple, as helical thickenings are a common identifying feature for the *Acer* genus (Figure 1). This species is a hardwood that is commonly found close to riparian forests in the Pacific Northwest.

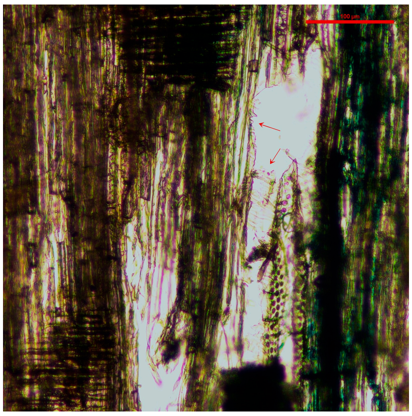

Figure 1. Radial section of a big leaf maple with red arrows pointing the helical thickenings on the vessel cell walls. Magnification 200×.

One specimen was identified as willow (*Salix* spp.). The sample presented both procumbent and upright rays. The rays were exclusively uniseriate (Figure 2), and the vessel cell walls presented pitting (Figure 3), which are all identifying features of *Salix* sp. L. The participants incorrectly identified this sample as a maple in the field.

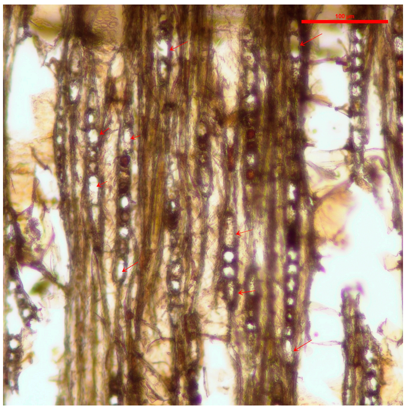

Figure 2. Tangential section of a willow with arrows pointing to the upright ray cells. All rays are uniseriate. Magnification 200×.

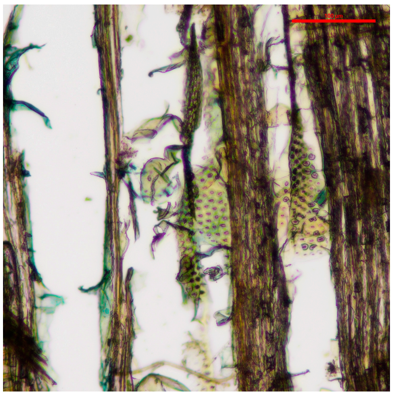

Figure 3. Radial section of a willow showing alternate vessel wall pitting and *Chlorociboria* spp. pigmentation. Magnification 200×.

Wood species identification in Peru was generally not successful. Those logs containing blue-green pigments were often too decayed to even attempt a genus identification. The participants were not able to do any in-field wood identification at this site.

The identification of the wood species is not required for citizen-scientists, but, if such people are capable, it is a nice addition to the data.

3.4. Verification

Performing the sampling under supervision and further verification showed that the method was effective. Therefore, the collection method can be done by citizen-scientists without professional supervision, as it has shown to be accurate.

4. Discussion

4.1. Training, Sampling and Sample Collection

In terms of simple mapping of *Chlorociboria* distribution, the training was deemed successful as eight logs containing *Chlorociboria* spp. were identified within the McDonald-Dunn and Mary's Peak Forests, and twelve logs suspected to contain *Chlorociboria* spp. were found in Madre de Dios, Peru, and all were successfully identified by the participants. Wood identification was less successful, unfortunately, especially in the tropical rainforests of Peru. It seems likely that this aspect of the proposed citizen scientist survey for *Chlorociboria* would generally not be useful, as researchers would have no way to verify correct wood ID, and when there is an abundance of diversity, the likelihood of misidentification is great.

A key element that allowed for the successful identification of the logs containing or suspected to contain *Chlorociboria* spp. was opening the logs well into the sapwood to find the blue-green colored wood. This helped students discount fungi, lichens, and bacteria that can colorize the surface and the first few centimeters of wood, and thus limited them to general decay-capable fungi. Another important element that was unique to this study was that it allowed for fungi without fruiting forms to be surveyed (when said fungi have a unique coloration system, such as *Chlorociboria* spp.) [39]. This method could easily be applied to non-fruiting bodied, pigment producing fungi, such as *Scytalidium cuboideum*, which develops red pigmentation [27], and *Scytalidium ganodermophthorum*, which develops yellow pigmentation [56,57], although these fungi do not have 'unique' colorants. A final factor that aided in the accurate identification was likely the focus on a single, distinct genus, allowing the participants to focus their attention. Surveys asking for a wider number of specimens to be located risk over or underestimation [4,58,59].

Another useful aspect of this technique was the use of common and accessible surveying tools, such as machetes (which can be easily replaced by hatchets and/or field knives), measuring tapes, smart phone GPS applications and/or GPS. These tools require little to no training for their use, and are generally accessible around the world. This is important for future reliance on citizen-scientists for the evaluation of *Chlorociboria* spp. in the wild, and should be effective. Previous studies have shown citizen science to be very useful, through works like eBird for data collection of bird diversity and migration patterns, which fully relies on bird hobbyists around the world [5,6]. Specific to mycology, the active participation of Pacific Northwest mycological societies in the ten-year chanterelle project resulted in the evaluation harvesting techniques of wild chanterelles in Oregon [8–10]. Similarly, the long-term survey in Warwickshire in the UK from 1965 to 1980 gave one of the most detailed surveys for fungi in the UK, and was performed by lay mycologists of the area [7].

As with other citizen scientist surveys, the technique detailed herein is similar to a rapid assessment survey (RAPD). This method is commonly used for field surveys by non-specialized personnel in specific areas [60]. The rapid assessment focuses on a group of genera in small, diverse areas. RAPD has been widely used for mycological surveys, but, if used for a wide number of genera, can become unreliable [58]. Focusing on a single group increases its accuracy. The modified RAPD method proposed herein has been shown to be accurate across different ecosystems (temperate rainforest in Benton County, OR, USA and the tropical rainforest in Madre de Dios, Peru) for the identification of the *Chlorociboria* spp. on downed wood by non-specialized personnel. In the future, this method could be applied by citizen-scientists to collect data on the worldwide distribution of the genus *Chlorociboria*.

The method proposed can also be applied without setting a transect, as it was performed in the Amazon rainforest. This variation can improve the participation of citizen-scientists, as some of them are mycology aficionados that can mark the location of *Chlorociboria* spp. while on their foraging activities, or simply on general hikes.

The distribution data (GPS points), which could be easily uploaded into a worldwide database such as GBIF, allowed the researchers on this project to identify forest types using Forest Service maps and forest data from the Peruvian government. This allowed for generation of a distribution map and an understanding of forest types preferred by *Chlorociboria* species. Continuation of this survey by citizen scientists could greatly expand our understanding of the types of trees and systems the genus of fungi prefer, even if exact tree species cannot be reliably noted. This data could then be made available to forested landowners seeking to manage for *Chlorociboria* species, xylindein production, and the economic opportunities therein.

4.2. Future Research

Currently, the location of the species is determined with a GPS app, and field notes. In the future, the development of a smartphone application (like eBird [5,6]) specific to the distribution of *Chlorociboria* spp. will be necessary to make the sampling, data collection, and data analysis simpler. This would also give the researchers involved one main input point for data, making analysis and map generation easier.

As for the implementation of this initiative, the unique laws and access to natural landscapes in different countries must be taken into account if the final goal is to apply it worldwide. In the immediate future, the sampling of *Chlorociboria* spp. can be done in association with mycology societies from the Pacific Northwest. This can help to improve the location method and to develop the application with them, as they have already participated in long-term studies (such as the Chanterelle project) [10]. In the intermediate future, contacts with other mycological societies could be done on a worldwide scale (such as with the Danish Mycological Society). In the long-term, the authors hope that this method might be introduced into countries that do not have a culture of foraging (like Peru).

Finally, the goal of the developed method and initiative is to gather data that can generate a detailed map of distribution of *Chlorociboria* spp., and, with it, the identification of areas that present a more frequent presence of the fungus. This information will be evaluated to determine the type of forests and the dominant wood species in those areas, and to then apply it to land management for the growth of these rare and useful fungi, should this be of interest to landowners.

5. Conclusions

The proposed method utilized simple tools and a smart phone or GPS to survey populations of *Chlorociboria* spp. across temperate and tropical forests. The method for identification of *Chlorociboria* spp. containing wood was shown to be successful; however, the wood identification was not. However, in general, *Chlorociboria* identification can be easily taught to members of mycological societies, eco tourists, and other interested parties that wish to be involved in recording the distribution of *Chlorociboria* spp. Over time, the data collected could be used to build a worldwide picture of forest type and wood preference by *Chlorociboria* species, which will be of use to those who wish to manage for xylindein production on their forested lands.

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