Field Methods for the Detection of *Bridgeoporus nobilissimus* DNA in Trees, Stumps, and Snags

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Background

In previous work *Bridgeoporus nobilissimus* DNA was successfully detected in wood samples taken from stumps and snags colonized by this fungus, using the specific primers described by Redberg et al[1]. *B. nobilissimus* conks are associated with the root crowns and lower trunks of large noble fir (*Abies procera*). The purpose of the study presented here was to continue this work by developing an efficient survey method for *B. nobilissimus*, and by doing limited surveys of unknown trees at *B. nobilissimus* sites.

Although the conks produced by *B. nobilissimus* are usually found on stumps and snags, they are occasionally found on living trees. By targeting these living host trees and analyzing the location of the fungus within these trees, we hoped to efficiently direct the sampling of unknown live trees. Stumps and snags with *B. nobilissimus* conks were also sampled.

The specific questions addressed in this study are:

- In taking wood samples for fungal DNA analysis, where is the best location on the tree to sample?
- Shallow samples are easier to obtain than samples deep within the tree trunk. What is the minimum depth that can be sampled and still detect *B. nobilissimus* if it is present?
- Can a standard increment borer be used efficiently to obtain samples, and how can it be cleaned between samples to prevent cross-contamination?
- *B. nobilissimus* DNA has been detected in stumps and snags with a *B. nobilissimus* conk. Can *B. nobilissimus* DNA be detected in live trees with a conk?
- Can *B. nobilissimus* DNA be detected in trees or stumps that have no *B. nobilissimus* conk, but are growing in the vicinity of a tree or stump with a *B. nobilissimus* conk?

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Materials and Methods

Study Sites

Four sites on BLM (Salem District) and USFS (Mt. Hood National Forest and Willamette National Forest) were chosen by the agencies for study. Table I lists the sites and the types of trees that were sampled at each site. The “unknown” stumps, snags, and live trees were true firs lacking a *B. nobilissimus* conk that were within 20 m of a host tree, so the *B. nobilissimus* status of these trees is unknown.

[1] Redberg, et al., 2003. Phylogeny and genetic diversity of *Bridgeoporus nobilissimus* inferred using mitochondrial and nuclear rDNA sequences. *Mycologia,* 95(5), 2003, pp. 836–845.

Table 1 Study sites and types of trees sampled at each site.

Site	Stumps/ Snags with BRNO Conk	Live Trees with BRNO Conk	Unknown Stumps/ Snags	Unknown Live Trees
Snow Peak 1 Salem BLM	1	2*	0	0
Snow Peak 2 Salem BLM	1	0	3	2
Gordon Mdw. Willamette NF	2	0	0	4
Goat Mtn. Salem BLM	1	1	1	2
South Fk. Mtn. Mt. Hood NF	0	1	1	3

*One of the "live trees with BRNO conk" sampled at Snow Peak 1 was recently cut, but clearly had produced a conk while still alive and thus was considered more representative of a live tree than a stump.

In addition, quality control samples were taken at each site to check the efficacy of the sampling tool cleaning method.

Tree Sampling Method

Samples were taken with an 18" increment borer. The tool was used as it typically is in removing a wood core for tree ring analysis. When the wood core was removed, it was split into three pieces: an outer piece, middle piece, and an inner piece. The three pieces were put into separate pre-labeled zip lock bags. Because samples were placed in a cooler and kept at 5º C, storing the samples in plastic was acceptable rather than the usual practice of storing moist wood cores in a breathable material such as paper. After every wood core was taken, the increment borer was cleaned to prevent cross-contamination. The cleaning procedure was as follows: First the auger and quill extractor were agitated in a wash solution, a 1% aqueous solution of the surfactant Triton X-100. Then the auger and extractor were placed in disinfecting solution, a 1% solution of Triton X-100 in isopropyl alcohol. The extractor was then wiped with a clean dry cloth, and the inner surface of the auger was cleaned with a .22 rifle cleaning tool. This tool consists of a rod with a slotted end. A small fabric cleaning patch is inserted in the slot, and the rod is pushed up and down inside the auger. A new cleaning patch must be used for each cleaning. Finally the outside of the auger was dried with a clean dry cloth. The cleaning solutions were

kept in 1 liter bottles with tight-fitting tops so they could easily be transported in a backpack to sampling sites.

The efficacy of the cleaning method was tested by taking "blank" cores at each of the study sites. Blank cores were taken from *Tsuga heterophylla* trees after sampling a *B. nobilissimus* host tree and cleaning the increment borer using the method just described. *Tsuga heterophylla* is not known to be a host for *B. nobilissimus*.

On the first site visit, samples were taken at various heights on the tree or stump. Analysis of this data indicated that the lower samples (ground level to 0.5m above ground level) were the most effective at capturing *B. nobilissimus* DNA in host trees. Based on this, all subsequent samples were taken at this lower level. After the initial site visit, four cores per tree or stump were taken, except for one live tree with a *B. nobilissimus* conk (Goat Mtn.), where 8 samples were taken. All depths of the cores from trees or stumps with a *B. nobilissimus* conk were tested. On unknown trees, only the inner and outer samples were tested. On some extremely rotted stumps and snags, the tree structure consisted of an outer shell with little or no material inside. On these subjects only 1 or 2 samples could be taken per hole.

Each live tree, snag, and stump sampled was labeled with a flasher tag. Tags were nailed to the base of the tree, on the downhill side using aluminum nails.

Lab Work

Samples were refrigerated until they were used. To prepare a sample for DNA extraction, a sample was removed from its bag and typically broken near the middle. About .2 to .3 g of sample was produced by chopping the core into small pieces using a sharp cutting tool. The wood pieces were then added to a labeled 2.0 ml microcentrifuge tube. DNA was extracted using a Chelex extraction buffer as follows. To each tube, 800 µl of Chelex buffer (100 mM Tris pH= 8.5, 8% Chelex 100 (Bio-Rad Laboratories), 1% Triton X-100) was added. About 20 1mm zirconia beads were added to the tube. Tubes were heated to 94ºC to 99ºC for 10 min., vortexed for 60 sec., and then returned to 94ºC to 99ºC for 10 min. Samples were then frozen, thawed, and centrifuged for 2 min. Each DNA sample was cleaned using a DNA clean up kit (UltraClean GelSpin DNA Purification Kit, MoBio Inc.) according to product directions.

Each cleaned DNA sample was run at least once in a PCR reaction. Reactions were 25 µl in volume and contained 1 U of JumpStart Taq (Sigma), 1X of Taq buffer, 200 µM each dNTP, 400 nM each specific primer, and 5.0 µg Bovine Serum Albumin (Biotechnology Grade, Amresco). Reactions were subjected to 94ºC for 1.5 m, followed by a touchdown PCR routine, where the anneal temperature varied from 66ºC to 62º C for the first five cycles, and was set at 61ºC for 40 cycles. Anneal time was 50 sec. Extension was 72ºC for 60 s, and denaturing was at 94ºC for 25 sec. Products were visualized on an agarose gel and photographed. Samples that showed a distinct 550 bp band were scored as positive.

To ensure that contamination was not taking place during lab processing of samples, every 20th extraction was a negative control. These controls went through the extraction process, but contained no wood sample. Every PCR batch contained at least one negative PCR control and one positive PCR control.

Results

A total of 257 samples were extracted and tested from the 5 sites, including 10 quality control samples. The results of PCR amplification were generally very clear, with either a strong amplicon of the expected size or no products at all. Few extraneous bands were produced. Figure 1 is a typical result.

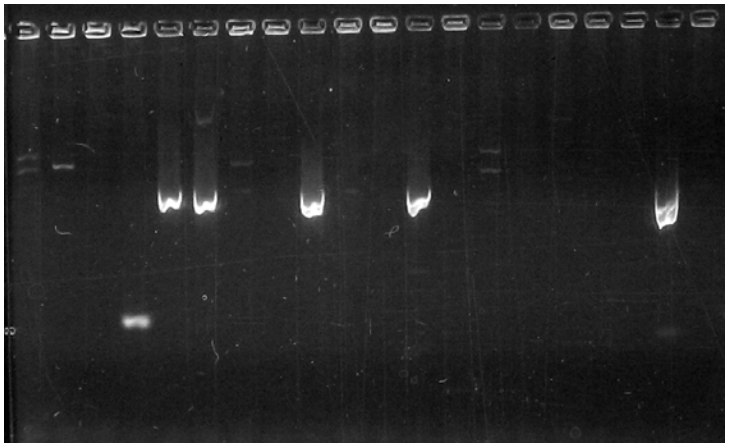

Figure 1. Results of PCR and gel on DNA extracted from wood samples. The amplicon to the far right is from DNA extracted from a *B. nobilissimus* sporocarp.

The results for each sample are given in the spreadsheet "Brno Field Methods Results". The results are summarized in Tables II-XIII where each tree is identified by an alphanumeric site code and a unique tree number. Site codes are as follows: 1) Snow Peak 1 is B, 2) Snow Peak 2 is C, 3) Gordon Meadow is D, 4) Goat Mtn is E, and 5) South Fork Mtn is F.

Sample Height Results

During the first sampling visit, samples were taken at different heights on the trees, which included two live trees with a *B. nobilissimus* conk and one snag with a *B. nobilissimus* conk.

Table II
Results for Lower and Upper Samples, Snow Peak I

Snow Peak I	# samples	Lower Samples	Upper Samples

Trees		% positive	% positive
All Trees (3)	45	44%	25%
Live Trees (2)	33	33%	13%

Results for All Sites, Sorted by Tree Type

The following tables summarize the data from all the sites by tree type. The four tree types are: dead trees with a *B. nobilissimus* conk, live trees with a *B. nobilissimus* conk, unknown dead trees, and unknown live trees. Dead trees include stumps and snags, and all unknown trees were within 20 m of a *B. nobilissimus* host tree. The percentage of cores with a positive sample is given to indicate the sampling effort needed to detect *B. nobilissimus* when it is present.

Table III
Stumps and Snags with *B. nobilissimus* Conk

Tree	# samples	# positive samples	% positive cores
B3	12	67%	79%
C1	12	92%	100%
D3	10	80%	100%
D7	9	11%	25%
E1	6	0%	0%

Table IV
Live Trees with *B. nobilissimus* Conk

Tree	# samples	# positive samples	% positive cores	% positive cores lower only
B1	24	25%	62%	75%
B2	9	22%	33%	50%
E3	24	29%	62%	62%
F5	12	67%	100%	100%

Table V
Unknown Stumps and Snags

Tree	# samples	# positive samples	% positive cores
C3	9	22%	25%
C5	8	100%	100%
C6	8	12%	25%
E4	8	0%	0%
F3	9	11%	25%

Table VI
Unknown Live Trees

Tree	# samples	# positive samples	% positive cores
C4	8	0%	0%
C7	3	0%	0%
D1	8	0%	0%
D2	9	22%	50%
D4	8	0%	0%
D6	8	0%	0%
E2	8	38%	75%
E5	8	12%	25%
F1	8	12%	25%
F2	8	25%	25%
F4	8	12%	25%

Depth of Samples and Results for Different Types of Trees

The final column in each table below gives the number of positive trees identified as positive considering only the samples taken at the indicated depth. This indicates the effectiveness of sampling at each depth.

Table VII
Results by Depth in Unknown Live Trees

Depth	# samples	% positive	Positive trees identified
Outer	42	12	4/6

Middle	11	0	0/6
Inner	31	16	5/6

Both outer and inner samples are needed to identify positive live trees.

Table VIII
Results by Depth in Unknown Dead Trees

Depth	# samples	% positive	Positive trees identified
Outer	21	38	4/4
Middle	9	33	1/4
Inner	12	8	1/4

The outer samples alone could identify these positive dead trees.

Table IX
Results by Depth in Live Trees with *B. nobilissimus* Conks

Depth	# samples	% positive	Positive trees identified
Outer	23	43	4/4
Middle	23	17	2/4
Inner	23	39	4/4

Either the outer or inner samples alone could identify the positive live *B. nobilissimus* trees.

Table X
Results by Depth in Dead Trees with *B. nobilissimus* Conks

Depth	# samples	% positive	Positive trees identified
Outer	25	60	4/4
Middle	16	38	2/4
Inner	8	88	3/4

Outer samples alone could identify the positive live *B. nobilissimus* trees.

Orientation of Samples and Results for Different Types of Trees

The final column in each table below gives the number of positive trees identified as positive considering only the samples taken at the indicated orientation. Orientation is coded as follows:

1 downhill side of tree
2 90º clockwise from 1
3 90º clockwise from 2 (uphill side)
4 90º clockwise from 3

Table XI
Results by Orientation in Unknown Live Trees

Orientation	# samples	% positive	% positive cores	Positive trees identified
1	20	5	9	1/6
2	20	5	9	1/6
3	21	19	27	3/6
4	21	19	36	4/6

Three different orientations of cores are needed to identify all the positive trees in this group (Table XI).

Table XII
Results by Orientation in Unknown Dead Trees

Orientation	# samples	% positive	% positive cores	Positive trees identified
1	11	18	20	1/4
2	7	14	25	1/4
3	10	10	20	1/4
4	9	33	50	2/4

Three different orientations of cores are needed to identify all the positive trees in this group (Table XII).

Table XIII
Results by Orientation in Live *B. nobilissimus* Trees

Orientation	# samples	% positive	% positive cores	Positive trees identified
1	12	50	75	3/4
2	9	67	100	3/4
3	12	25	75	3/4
4	9	56	100	3/4

Two different orientations of cores are needed to identify all the positive trees in this group(Table XIII).

Table XIV
Results by Orientation in Dead *B. nobilissimus* Trees

Orientation	# samples	% positive	% positive cores	Positive trees identified
1	11	36	60	3/4
2	9	67	60	3/4
3	11	63	80	4/4
4	13	62	50	3/4

All the positive trees in this group (Table XIV) could be identified using cores from orientation 3 (uphill side) alone.

Quality samples

A total of ten quality control samples were taken over the five study sites to test the efficacy of the increment borer cleaning method. This test is only meaningful if the sample taken just prior to the quality sample was positive. Table XV gives the results of each quality sample and each prior sample.

Table XV
Results for quality samples and the sample previous to the quality sample
(1 = positive result, 0= negative result)

Quality sample #	Previous sample #	Previous sample result	Quality sample result
BQ01	B391	1	0
BQ02	B3111	0	0
BQ03	B343	1	0
BQ04	B3121	1	0
CQ01	C113	1	0
CQ02	C123	1	0
DQ01	D733	0	0
DQ02	D712	0	0
EQ01	E383	0	0
FQ01	F513	1	0

Discussion

Previous work has shown the value of using a species-specific DNA marker to locate *B. nobilissimus* in dead trees. The work reported here was a pilot study mainly concerned with developing methods and testing sampling tool use, tool cleaning, and the distribution of the fungus within host trees. Another goal of this study was to determine if these sampling techniques could be used to locate *B. nobilissimus* in living trees, as this would open the door to accurately surveying for this fungus in potential hosts that showed no outward signs of inhabitation by the fungus. This type of DNA-based survey technique will allow the assessment of the true extent of populations of this fungus, give more accurate knowledge of host preferences, and more knowledge of the survival strategies of this fungus.

As expected, *B. nobilissimus* DNA was detectable in many of the samples taken from stumps and snags with a *B. nobilissimus* conk (Table III). For two *B. nobilissimus* host stumps, *B. nobilissimus* was detected in every sample. These results indicate that *B. nobilissimus* can be a prominent member of the guild of fungi colonizing noble fir stumps. The stump that had *B. nobilissimus* in only one of four cores contained fruiting bodies of several other fungi. The *B. nobilissimus* conk on this stump seemed to be in decline and had separated from the main body of the stump. It is unclear why stump E1 was negative in all six samples; it may have been outcompeted by other fungi that were not fruiting at the time of sampling. Even though *B. nobilissimus* was not detected in any of the samples taken from this stump, it was detected in three of the samples taken from the live noble fir growing next to it. *B. nobilissimus* DNA was also easily detectable in the four live trees with conks (Table IV). For samples taken near ground level, at least 50% of the cores were positive in all four of these trees.

Surprisingly, four of the five stumps and snags with no conk tested positive for *B. nobilissimus* DNA in at least one sample out of eight (Table V). Tree C5 was a 7” diameter snag within one meter of a host stump. All eight of the samples from this tree were positive. Apparently, many of the dead noble firs in the vicinity of known host trees are also colonized by *B. nobilissimus*, although they do not produce a conk. *B. nobilissimus* was also detected in six of eleven live trees with no *B. nobilissimus* conk (Table VI), again indicating that this fungus has significantly more hosts than is indicated by the presence of its fruiting bodies. The positive live trees included one 20” and one 14” diameter tree. This is significantly smaller than the typical diameter of trees and stumps that support *B. nobilissimus* conks, and indicates that the number of potential hosts for this fungus is much larger than previously thought. However, large diameter trees and stumps may be necessary for sporocarp production.

Each site had at least one “unknown” tree that was positive for *B. nobilissimus*. At the South Fork Mtn. site, three unknown live trees and one unknown snag were tested; all of these unknowns were positive. The presence of a *B. nobilissimus* sporocarp in a stand indicates that other trees besides the host tree are colonized by this fungus.

Location within the tree

Height

The analysis of the results from the first visit (Table II) indicated that *B. nobilissimus* could be detected in living trees, snags, and stumps with conks, and that positive cores were more likely to be obtained from samples taken lower on the tree. In subsequent sampling, all cores were taken low on the tree, from 0 to 0.5 m from ground level. These findings are in agreement with the hypothesis that this fungus is associated with noble fir root systems.

Depth

The effect of sample depth is shown in Tables VII to X. In three of the four tree types, the outer and inner samples are nearly equal in ability to detect *B. nobilissimus*, while the middle sample is not as effective. The outer sample was most effective at identifying positive trees, but this sample depth only identified eight out of ten positive unknown trees and stumps. It would be beneficial if positive trees could be identified from outer samples, since these require less effort to obtain cores. In this study, four 45 cm cores were taken from each tree. It may be worthwhile to explore whether six or eight 15 cm cores could be as effective at identifying positive trees.

Orientation

B. nobilissimus conks almost always appear on the downhill side of a host tree. Because of this, it was hypothesized that the mycelium may have a specific orientation within the tree as it colonizes its host. To test this, we defined an orientation scheme on each tree, with orientation 1 being the downhill side of the tree. Orientation 2 was 90º clockwise from 1, and so on. With this scheme, one could hypothesize that orientation 1 would be most effective at detecting *B. nobilissimus* when it is present. As Tables XI through XIV show, this is not the case. There does not seem to be any effect of orientation on the ability to detect *B. nobilissimus*.

For the unknown trees, at least three different core orientations were required to identify the positive trees. This indicates that the cross-sectional growth pattern of *B. nobilissimus* is irregular, and surveying for *B. nobilissimus* will involve taking core samples from multiple directions.

In one live tree with a conk, eight core samples were taken to better assess the distribution of the fungus in the tree. Of the eight outer samples, orientations 1, 2, and 4 were positive; none of the middle samples were positive; and inner samples 1, 1.5, 2, and 3 were positive. These results indicate a discontinuous distribution of the fungus in each cross section of the tree trunk. In this case there seems to be four patches of colonization, one at the outer part of the trunk near each of the locations 1, 2, and 4, and one patch in the middle of the tree. It has

been hypothesized that *B. nobilissimus* enters the host tree through the root system. In areas colonized by *B. nobilissimus*, individual trees may become infected independently through several major roots leading to the discontinuous interior colonization pattern seen here.

Cleaning Method Effectiveness

In six cases a quality sample was taken immediately after a positive sample. In all six of these cases the quality sample was negative (Table XV). These results indicate that the cleaning procedure described in this report is sufficient to prevent cross-contamination between samples taken with an increment borer.

Conclusions

The results of this study indicates that *B. nobilissimus* typically has more nearby hosts than the tree on which it produces a conk. *B. nobilissimus* may have the strategy of occupying many trees over a large area, but only producing one or few sporocarps that grow to a large size, rather than many smaller sporocarps.

It appears that the colonization pattern of this fungus within trees lacking a conk is irregular, with a concentration at the lower part of the tree, and in the outer and inner parts of the tree stem. The cross-sectional distribution is not even, and sampling from one direction is not likely to detect the fungus in all colonized trees. It seems that sampling unknown trees from at least three directions is needed, and more samples are recommended to decrease the chance of missing a positive tree.

At this time, a recommended protocol (Appendix 1) for testing unknown trees for the presence of *B. nobilissimus* would be as follows:

- Remove four core samples of 45 cm (or to the center of the tree), all cores taken within 0.5 m of the ground.
- Test the outer and inner part of each core.

The increment borer cleaning procedure documented here and in the document "Procedure for Sampling Tree Stems for Bridgeoporus nobilissimus DNA Analysis" is sufficient to prevent cross-contamination of samples.

Further Work

This study found *B. nobilissimus* present in trees with no conks at all four study sites. We did not test any trees farther than 20 m from a conk, so we do not know the extent of a *B. nobilissimus* colony at any site. To determine *B. nobilissimus* colony diameter, a survey along transects starting at a *B. nobilissimus* conk, and extending out 100 m or more may be effective and would be useful in defining the area to be protected around a *B. nobilissimus* conk.

One question is if *B. nobilissimus* is a rare fungal species. New *B. nobilissimus* sites could possibly be located and range and habitat gaps could be addressed by sampling likely host trees in potential habitat rated as having a high likelihood of supporting *B. nobilissimus* by habitat models or expert opinion.

It is not known how natural and human disturbances affect the *B. nobilissimus* colonies. One site (trees 1 and 2 at Goat Mtn.) was recently thinned. Though we do not know the colony extent prior to thinning, it may be of benefit to sample this site now to determine the extent of the colonization, and then sample at a later date to see if the level of colonization is stable, shrinking, or increasing. The results of such a study would give some insight into the effect of forest thinning on existing colonies of *B. nobilissimus*.

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Kelli Van Norman and Terry Fennell of the Oregon BLM defined the scope and objectives of this project. I appreciate the invaluable field assistance of botanists Terry Fennell, Alice Smith, and David Lebo.

Appendix 1

Procedure for Sampling Tree Stems for *Bridgeoporus nobilissimus* DNA Analysis

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This document provides a method for collecting wood samples that can be used to obtain the DNA of symbiotic fungi that may be inhabiting the tree or snag from which the wood was taken. The method works well on living or recently dead trees, stumps, and snags. Wood that has decayed for a time will not provide enough cohesion to allow the increment borer auger to bite into the wood. In this case, samples can be removed by hand or with a knife.

Tools and Materials

- Increment borer, 18", with .25" internal diameter
  A smaller increment borer can be used, but if the auger chamber has a smaller diameter, the cleaning tool must be sized appropriately.
- Rifle cleaning rod (.22) with slotted end, with small fabric cleaning patches.
- Aqueous solution of Triton X-100(1%), minimum size 1 liter.
- Isopropyl alcohol solution of Triton X-100 (1%), minimum size 1 liter.
- Two containers for the solutions tall enough to hold the auger and extractor.
- Clean dry cloth for drying
- Recloseable plastic bags for holding core samples
- Permanent marker pen
- Cooler with ice or cooling block

Procedure

Preliminary work indicates that the *B. nobilissimus* fungus is more often detectable lower on the trunk of the tree. Use the increment borer according to standard methods to remove a core within 0.5 m of ground level. When starting to bore, position the auger to maximize the depth that can be achieved. It is sometimes beneficial to angle the auger slightly downward, but

too much of an angle prevents the full depth from being reached because the tree trunk eventually prevents the handle from rotating. The core can be broken into subsamples, or kept intact as one sample. Place each sample into a labeled zip-lock plastic bag and label each bag with permanent marker pen indicating the appropriate tree and sample location information. If the ambient temperature is over 10º C, place the sample bag in a cooler.

The increment borer must be cleaned and sterilized. If the purpose of the survey is to determine which trees are colonized by *B. nobilissimus*, the borer needs to be cleaned after all the samples from one tree have been taken. If the purpose is to determine where in the tree the fungus is located, the borer must be sterilized after each core sample.

To sterilize, place the auger and extractor in the wash solution, the aqueous solution of Triton X-100, and agitate gently. The purpose of this step is mainly to remove the shreds of bark that tend to cling to the outside of the auger. Remove the tools from the wash solution, let them drain a few seconds, then place them in the Triton X-100/ isopropyl alcohol solution and agitate gently. This is the disinfection step. Allow the auger to soak in the disinfection solution while you insert a fresh cleaning patch into the slotted end of the gun cleaning tool. Remove the auger and allow it to drain back into the bottle, then run the cleaning tool up and down the auger bore. Finally, dry the outside of the auger and the extractor with a clean dry cloth. At this point, the borer is ready to take another sample.

During transportation and storage the samples should be kept between 0º to 5º C. Wood samples have been stored successfully (*B. nobilissimus* DNA still detectable) for over 1 year under refrigeration. Freezing will preserve the DNA within the sample, but repeated freeze/ thaw cycles will degrade DNA, and should be avoided.