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ORIGINAL ARTICLE

Optimization of sampling procedures for DNA-based diagnosis of wood decay fungi in standing trees

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Abstract

Aims: To develop fast and reliable sampling procedures for DNA-based diagnosis of wood decay fungi in standing trees.

Methods and Results: A total of 250 trees were tested for the presence of a suite of wood decay fungi by collecting wood frass obtained by drilling each tree once with a 4-mm-diameter, 43-cm-long bit. We identified at least one of 11 target wood decay fungi in 56 trees through multiplex PCR assays. The presence of target wood decay *taxa* was further investigated in these 56 trees, by analysing independently wood from each of six drillings. Results were then compared with those obtained using sampling schemes differing in terms of number and position of drillings. Samples of 1–4 drillings were either analysed separately, and the results were combined, or pooled together before analysis was performed. In comparison with *taxa* identified by the analysis of six drillings, diagnostic efficiency ranged from 56·6% for the scheme based on a single drill to 96·8% for the scheme based on four drillings analysed separately. Both schemes significantly differ ($P < 0{\cdot}05$) from those based on two and three drillings, whose efficiency was 72·6% and 83·9%, respectively. Diagnostic efficiency of pooled samples was comparable to that of samples analysed separately.

Conclusions: Highest diagnostic efficiency was obtained by analysing wood from four drillings. It is advisable to pool samples deriving from different drillings to reduce laboratory costs.

Significance and Impact of the Study: Fast and reliable sampling procedures make DNA-based diagnosis more suitable for tree inspection procedures.

Introduction

Taxon-specific multiplex PCRs have been recently developed for the timely detection and identification of several wood decay fungi deemed to be hazardous for tree stability (Guglielmo *et al.* 2007, 2008b; Nicolotti *et al.* 2009). DNA-based techniques can accurately diagnose rotting fungi directly from wood and represent a straightforward alternative to traditional identification methods, which mostly rely on the occurrence of fruit bodies and/or the isolation of pure fungal cultures (Nobles 1965; Stalpers 1978; Breitenbach and Kränzlin 1986; Bernicchia 2005).

A proper assessment of the stability and integrity of trees is essential for the prevention of dangerous windthrows or limb failures. Because the structural deterioration of wood caused by rotting fungi can increase the occurrence of tree failures, the timely detection of wood decay is relevant in the assessment of hazardous trees. The biology and ecology of different rotting fungi are varied; hence, the knowledge of the fungal species involved is important for risk prediction (Lonsdale 1999).

Tree inspection procedures are currently relying on a primary visual observation of any evidence of structural weakness or decay, often followed by analyses using instruments aimed at assessing localization and extent of the decay (Mattheck and Breloer 1992). Penetrometers or tomographic techniques are frequently used during tree inspections (Tomikawa *et al.* 1990; Müller *et al.* 2001; Nicolotti *et al.* 2003).

In the absence of external signs and/or structural defects, trees tend to be categorized as not significantly hazardous (Lonsdale 1999). Nonetheless, most decay agents in the early and intermediate stages of infection can be overlooked owing to the absence of any visible signs of their presence. This can represent a serious problem in case of either rapidly progressing decay fungi that can turn a sound tree into a hazard in a short period of time or undetectable fungi present underground in the root system. To improve the accuracy of tree inspections, rapid and sensitive diagnostic methods, such as multiplex PCRs, may be helpful in detecting hazardous rotting fungi (Guglielmo *et al.* 2008a). A critical unresolved issue is on how to optimize sampling design to maximize the diagnostic power of DNA-based and other assays.

According to the sampling theory applied to tissue-based diagnosis, the probability of detection of any 'object' depends upon the 'basic space', the number and size of the samples collected (diagnostic frame) and the 'number' and 'size' of the objects to be detected (Kayser *et al.* 2009). When applying these concepts to the assessment of tree stability, 'objects' correspond to hazardous decay fungi to be detected in the 'basic space' that is represented by those parts of the tree, such as basal stem and roots, that bear particularly high static loads. Because the decay process in a living tree is the result of biological processes involving fungal and host responses mediated by environmental factors, the abundance and distribution of decay fungi in wood are inevitably patchy and varied (Rayner and Boddy 1986; Boddy 2000; Schwarze *et al.* 2004; Terho *et al.* 2007; Terho and Hallaksela 2008; Deflorio *et al.* 2009). Accordingly, the 'number' and 'size' of collection samples may greatly affect the probability of detection of decay fungi in trees.

The main goal of this work was to compare the diagnostic efficiency of drilling-based sampling schemes that differ in terms of number and position of sampling points on trees when using a recently developed multiplex PCR–based method (Guglielmo *et al.* 2007, 2008b; Nicolotti *et al.* 2009). For this purpose, we compared results obtained from six samplings with those based on one, two, three and four samplings. A second goal was to test whether the diameter of the tree can affect the diagnostic efficiency of the sampling schemes here described.

Materials and Methods

Sampled trees

A total of 250 trees located in and around the city of Turin, and encompassing uprooted trees, trees with evidence and suspect of decay (Table 1), were sampled by drilling a hole at the base of the trunk. The diameter at breast height (DBH, 1·3 m aboveground) was measured for each tree.

Sampling schemes

A 4-mm-diameter, 43-cm-long bit was used to drill trees at the base of the stem, *c.* 5 cm aboveground. The bit, made of chrome vanadium steel with a cobalt/tungsten carbide tip, was expressly manufactured for this study by Elli Zerboni (Turin, Italy) and was used in a drill with a maximum speed of 1400 rev min^{-1}. Wood chips generated during the drilling process were collected in a 9-cm-diameter plastic Petri dish and used as substrate for the DNA-based assay. After each sampling, the drill bit was carefully cleaned with a 0·5% (w/v) sodium hypochlorite solution, rinsed with sterilized water and wiped with ethanol 95% (v/v). After samplings, Thiophanate-methyl fungicide 38·5% (w/v) was sprayed on holes to prevent fungal infections. Samples were frozen at −20°C for at least 24 h, before being placed in a lyophilizer for an additional period of 24 h.

On the subsample of trees found to be positive to a fungal *taxon* identifiable through multiplex PCR–based method (Table 2), we tested eight sampling schemes differing in terms of (i) number of drillings performed and (ii) whether wood from each drilling was pooled before analysis (Fig. 1). Each tree was drilled either once, twice, three or four times with direction of drillings located at 180°, 120° and 90° from one another (Fig. 1). As a consequence, a total of six different drillings were performed per each tree (Fig. 1). In the case of four drillings, we analysed the samples in three ways: (i) all samples were analysed independently, (ii) all samples were pooled before analysis and, (iii) two samples were obtained by combining opposite drillings and each of the two independently analysed (Fig. 1).

Sample preparation and multiplex PCRs

For each drilling, 100 mg of lyophilized wood chips were collected in a 2-ml screw-cap tube containing two glass beads, one measuring 6 mm in diameter and the other with a 2 mm diameter. Each sample was then homogenized in a FastPrep FP120 Cell Disrupter (Thermo Electron Corporation, Milford, MA, USA) by shaking for 30 s

Table 1 Type, number, species and diameter of trees used to test the drilling-based method and the sampling schemes, as well as wood decay fungi identified through multiplex PCRs

Tree type	No of sampled trees	No of trees positive to multiplex PCRs *taxa**	Tree species	Diameter (cm)†	Fungal *taxa* identified through multiplex PCR
Uprooted trees	6	5	*Aesculus hippocastanum*	102	*Pleurotus* sp.
			Platanus hybrida	60	*Ganoderma resinaceum*
			P. hybrida	35	*Perenniporia fraxinea*
			Tilia hybrida	60	*Ganoderma adspersum*
			Ulmus sp.	70	*Armillaria* sp.
Standing trees with emerging fungal fruit body	32	21	*Acer pseudoplatanus*	40	*Armillaria* sp.
			A. hippocastanum	55	*G. resinaceum*
			Betula pendula	45	*G. adspersum*
			Carpinus betulus	30	*Armillaria* sp. + *Ganoderma* sp.
			Celtis australis	70	*G. resinaceum*
			C. australis	65	*G. resinaceum*
			C. australis	64	*G. resinaceum*
			C. australis	63	*G. resinaceum*
			C. australis	63	*G. resinaceum*
			C. australis	58	*G. resinaceum*
			C. australis	70	*P. fraxinea*
			C. australis	55	*P. fraxinea*
			C. australis	82	*G. resinaceum* + *P. fraxinea*
			C. australis	65	*G. resinaceum* + *P. fraxinea*
			Fagus sylvatica	140	*Armillaria* sp. + *G. resinaceum* + *Phellinus* s.s.
			Fraxinus excelsior	55	*Armillaria* sp.
			Quercus robur	125	*Armillaria* sp.
			Q. robur	105	*Fomitiporia* sp.
			Q. robur	45	*Fuscoporia* sp.
			Q. robur	100	*Fuscoporia* sp. + *P. fraxinea*
			Q. robur	130	*Laetiporus sulphureus* + *P. fraxinea*
Standing tree with suspect of basal decay‡	212	30	*A. hippocastanum*	60	*Inonotus/Phellinus*§
			A. hippocastanum	55	*Inonotus/Phellinus*
			A. hippocastanum	58	*Armillaria* sp.
			Platanus acerifolia	121	*P. fraxinea*
			P. acerifolia	74	*P. fraxinea*
			P. hybrida	110	*Armillaria* sp.
			P. hybrida	100	*Armillaria* sp.
			P. hybrida	86	*Armillaria* sp.
			P. hybrida	84	*Armillaria* sp.
			P. hybrida	82	*Armillaria* sp.
			P. hybrida	81	*Armillaria* sp.
			P. hybrida	67	*Armillaria* sp.
			P. hybrida	45	*Armillaria* sp.
			P. hybrida	40	*Armillaria* sp.
			P. hybrida	36	*Armillaria* sp.
			P. hybrida	31	*Armillaria* sp.
			P. hybrida	77	*Fomitiporia* sp.
			P. hybrida	58	*Fomitiporia sp.*
			P. hybrida	82	*G. resinaceum*
			P. hybrida	64	*G. resinaceum*
			P. hybrida	70	*Inonotus/Phellinus*
			P. hybrida	64	*Inonotus/Phellinus*
			P. hybrida	57	*Inonotus/Phellinus*
			P. hybrida	51	*Inonotus/Phellinus*
			P. hybrida	49	*Inonotus/Phellinus*

Table 1 (Continued).

Tree type	No of sampled trees	No of trees positive to multiplex PCRs *taxa**	Tree species	Diameter (cm)†	Fungal *taxa* identified through multiplex PCR
			P. hybrida	34	*Inonotus/Phellinus*§
			P. hybrida	76	*L. sulphureus*
			P. hybrida	90	*P. fraxinea*
			P. hybrida	66	*P. fraxinea*
			P. hybrida	53	*P. fraxinea*

*Trees infected by fungal *taxa* identifiable through multiplex PCRs (Guglielmo *et al.* 2007, 2008b) (for details see in the text).
†Diameters were measured at the breast height.
‡Suspect of decay was gathered from reports of professional arborists.
§Species belonging to either *Inonotus* or *Phellinus* but not identifiable at the subgeneric level using this DNA-based assay.

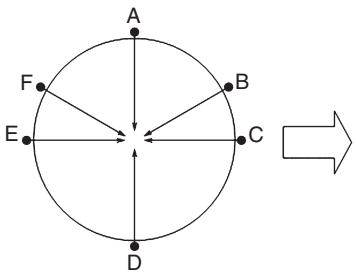
Table 2 Multiplex PCRs used in this study with related primers and *taxa* identified

	Primer combination		
Multiplex PCR	Forward	Reverse	*Taxon* identified (corresponding amplicon size)
M1	ITS1-F*	ITS4†	Fungi (*c.* 500–900 bp)‡
		Gano2R§	*Ganoderma* spp. (226–228 bp)
	F115§	Hyme2R§	*Inonotus/Phellinus* spp. (111 bp)
M2	ITS3†	Armi2R§	*Armillaria* spp. (185 bp)
	25sF§	LaetR§	*Laetiporus sulphureus* (146 bp)
		Pleu2R§	*Pleurotus* spp. (158 bp)
		Heri2R§	*Hericium* spp. (200 bp)
M3	ITS3	PerR§	*Perenniporia fraxinea* (152 bp)
		Schi2R§	*Schizophyllum* spp. (190 bp)
		Ste2R§	*Stereum* spp. (231–236 bp)
		Ustu2R§	*Kretzschmaria deusta* (260 bp)
	MS1†	TraR§	*Trametes* spp. (220 bp)
Mgano	ITS1-F	GadR¶	*Ganoderma adspersum* (211 bp)
		GapR¶	*Ganoderma applanatum* (200 bp)
		GlR¶	*Ganoderma lucidum* (193 bp)
		GrR¶	*Ganoderma resinaceum* (178 bp)
Mhyme	25sF	FomR¶	*Fomitiporia* spp. (258 bp)
		FuscR¶	*Fuscoporia* spp. (225 bp)
		IdryaR¶	*Pseudoinonotus* spp. (254 bp)
		InocuR¶	*Inocutis* spp. (265 bp)
		InssR¶	*Inonotus sensu stricto* spp. (214 bp)
		PhssR¶	*Phellinus sensu stricto* (173 bp)

*Universal fungal primers for ITS rDNA amplification whose nucleotide sequence is reported in Gardes and Bruns (1993).
†Universal primers for ITS rDNA amplification whose nucleotide sequence is reported in White *et al.* (1990).
‡ITS1-F and ITS4 primers allow for the nonspecific amplification of fungal rDNA whose size varied according to the fungal species.
§*Taxon*-specific primers whose nucleotide sequence is reported in Guglielmo *et al.* (2007).
¶*Taxon*-specific primers whose nucleotide sequence is reported in Guglielmo *et al.* (2008b).

at 4·5 m s^{-1}. Pooled samples were prepared by adding equal amounts of lyophilized wood chips derived from each drilling up to a final amount of 100 mg. In some trees, positive results were obtained when wood from each drilling was analysed independently, but results from pools of multiple drillings were negative. In these cases, we tested whether results could be improved by homogenizing the wood samples before pooling them. This pre-homogenization was obtained by freezing wood chips with liquid nitrogen and grinding them with mortar and pestle.

DNA extraction was performed using the QIAamp DNA Stool Mini kit (Qiagen, Valencia, CA, USA), following manufacturer instructions.



Sampling scheme code	Number and position of drillings	Samples to be analyzed per tree
1·1	1 Drilling (A or B or C or D or E or F)	1
2·1	2 Drillings at 180° (A – D or C – E)	1 (chips from 2 drillings at 180° mixed in 1 sample)
2·2		2 (chips from 2 drillings at 180° analyzed separately)
3·1	3 Drillings at 120° (B – D – F)	1 (chips from 3 drillings at 120° mixed in 1 sample)
3·3		3 (chips from 3 drillings at 120° analyzed separately)
4·1	4 Drillings at 90° (A – C – D – E)	1 (chips from 4 drillings at 90° mixed in 1 sample)
4·2		2 (chips from 2 opposite drillings mixed in 2 samples)
4·4		4 (chips from 4 drillings at 90° analyzed separately)
6·6	6 Drillings (A – B – C – D – E – F)	6 (chips from 6 drillings analyzed separately)

Figure 1 Transversal section of the base of a tree with the position of the drillings performed (indicated by the letters A, B, C, D, E and F) and a summary of the eight sampling schemes tested. The identifier of each sampling scheme (1.1, 2.1, 2.2, 3.1, 3.3, 4.1, 4.2 and 4.4) is composed by the number of drillings performed followed by the number of samples analysed. The sampling scheme 6.6 is the sampling scheme of reference with a 100% of diagnostic efficiency.

The multiplex PCR–based method recently summarized by Nicolotti *et al.* (2009) was used as DNA-based diagnostic assay. The multiplex PCRs named M1, M2 and M3 were performed on each sample, whereas the two further multiplex PCRs, named Mgano and Mhyme, were used to identify, at a subgeneric rank, species belonging to *Ganoderma* spp., *Inonotus* spp. and *Phellinus* spp. (Table 2). Each multiplex PCR was performed in a 25 μl volume containing 1× PCR buffer, 1·5 mmol l^{-1} of $MgCl_2$, 0·2 mmol l^{-1} of dNTPs mix, 0·5 μmol l^{-1} of each primer, 0·5 mg ml^{-1} of Bovine Serum Albumin, 0·025 U μl^{-1} of Taq polymerase (Promega, Madison, WI, USA) and 6·25 μl of a 1/50 dilution of wood DNA extract. PCRs were conducted using an initial denaturation at 94°C for 5 min, followed by 35 cycles with each cycle consisting of a denaturation at 94°C for 45 s, an annealing at a temperature ranging from 55 to 63°C, depending on the multiplex PCR (Guglielmo *et al.* 2007, 2008b), for 45 s, and an extension at 72°C for 45 s, and one final cycle with a 72°C extension for 10 min. Amplicons were visualized on a gel containing 1% (w/v) of high resolution MetaPhor (Lonza, Rockland, ME, USA) and 1% (w/v) of standard agarose (Applichem GmbH, Darmstadt, Germany), after a 2-h electrophoretic migration at 4 V cm^{-1}. The identification of a target *taxon* was inferred from the presence of an amplicon of specific size (Table 2). The absence of *taxon*-specific amplicons combined with the amplification of ITS (internal transcribed spacer) region with the universal fungal primers ITS1-F and ITS4, included in the M1 (Table 2), was regarded as the presence of fungi not identifiable through multiplex PCRs. No amplicon after multiplex PCRs was interpreted as absence of fungal DNA.

Data interpretation and statistical analyses

Diagnostic efficiency of each sampling scheme was calculated as the percentage of target *taxa* identified through multiplex PCR–based method (Table 2) by each sampling scheme with reference to the number of target *taxa* identified by analysing independently six drillings per tree. Lack of detection of any target *taxa* was regarded as a failed diagnosis, independently of whether fungal DNA was identified through the use of universal fungal primers, although lack of a universal fungal ITS band was regarded as a failure linked to DNA extraction. Diagnostic efficiency of each sampling scheme was also calculated separately for trees above and below 80 cm DBH. Finally, when more than one drilling was tested, we estimated diagnostic efficiency of pooled samples, with and without prehomogenization.

Yates' corrected chi-square or Fisher's exact test and fourfold tables were used to test differences among sampling schemes in terms of diagnostic efficiency. Differences were considered to be significant when $P < 0{\cdot}05$. Statistic analyses were implemented with SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

We identified at least one of 11 different target wood decay *taxa* through multiplex PCRs in 56 out of the 250 trees initially analysed with a single drilling (Table 1). All comparative results using one, two, three and four drillings were obtained from this subsample of 56 trees (Table 3).

Notwithstanding the results from the six independent drillings that were regarded as a term of comparison, rather than as a sampling option, the sampling scheme based on the preparation of separate samples from four drillings (4.4) had the highest diagnostic efficiency (96·8%). This value was the only one to be significantly higher ($P < 0{\cdot}05$) than those of all other schemes involving a lower number of drillings (Table 3). Sampling scheme 4.4, and 4.2, showed a diagnostic efficiency statistically undistinguishable from that obtained by pooling results from the six drillings analysed independently (6.6). Conversely, the

Table 3 Diagnostic efficiency (%) and failed diagnosis (%) of the eight sampling schemes. Diagnostic efficiency was expressed as the percentage of target *taxa* identified through multiplex PCR–based method with reference to the number of target *taxa* identified by analysing independently six drillings per tree

Sampling scheme code	Diagnostic efficiency (%)	Failed diagnosis (%)	
		Fungal DNA detected through the use of universal fungal primers*	Fungal DNA not detected†
1.1	56·6 a‡	29·3	14·1
2.1	73·0 b	23·8	3·2
2.2	79·4 bc	19·8	0·8
3.1	72·6 b	21·0	6·4
3.3	83·9 bcd	11·3	4·8
4.1	88·9 cde	11·1	0·0
4.2	92·1 def	7·9	0·0
4.4	96·8 ef	3·2	0·0
6.6	100·0 f	0·0	0·0

*Amplification of fungal ITS region with the primers ITS1-F and ITS4, but lack of detection of any target *taxa* identifiable through multiplex PCRs.
†No amplification of fungal ITS region with the primers ITS1-F and ITS4 and lack of detection of any target *taxa* identifiable through multiplex PCRs.
‡Yates' corrected chi-square or Fisher's exact tests. Relative frequencies followed by the same letter are not significantly different ($P > 0{\cdot}05$).

diagnostic efficiency of the one-drilling-based scheme was significantly lower than that of all the other schemes. The two- and three-drilling-based schemes showed intermediate diagnostic efficiency values and were statistically undistinguishable from one another. Diagnostic efficiencies were higher when drillings were analysed separately, but these differences were not significant (Table 3).

When tree size was taken into account, diagnostic efficiencies of most schemes were higher in trees below 80 cm DBH than in trees above 80 cm DBH, but differences were not statistically significant (Fig. 2).

Prehomogenization of wood samples, before pooling them for the analysis, increased diagnostic efficiency of the assay exclusively when a single drilling had resulted positive in the assay performed on individual drillings. Values of the diagnostic efficiency with and without prehomogenization were not significantly different.

Discussion

The sensitivity and accuracy of *taxon*-specific multiplex PCRs used in this study have been previously tested by

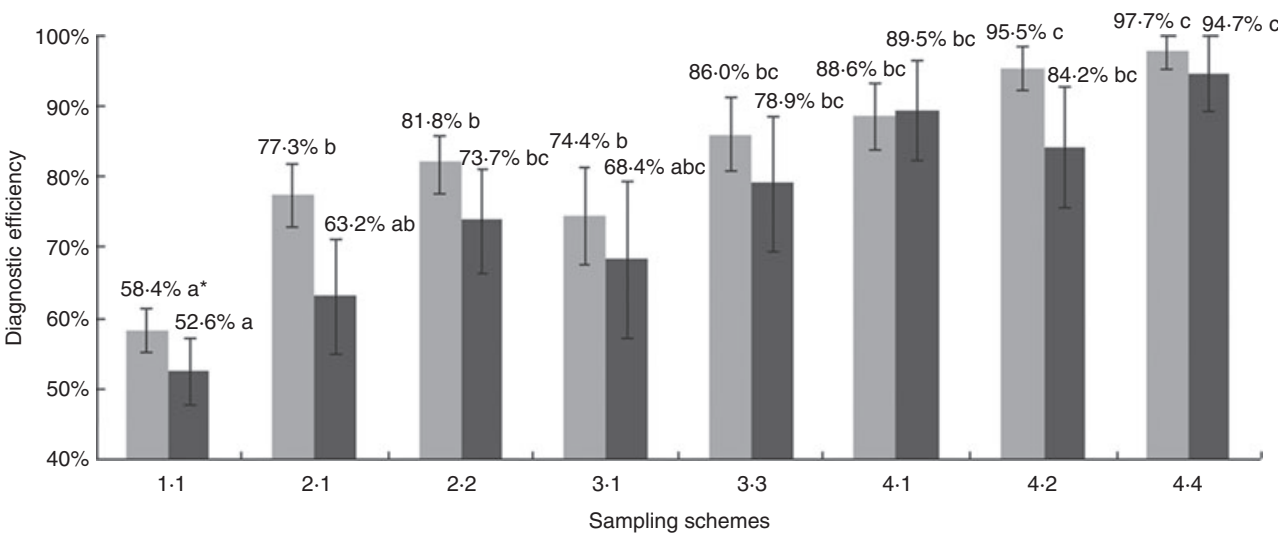


Figure 2 Diagnostic efficiencies of the sampling schemes on trees above and below 80 cm diameter at breast height (DBH). Error bars represent standard errors. (■) = DBH > 80 cm; (■) = DBH < 80 cm. *Yates' corrected chi-square or Fisher's exact tests. Relative frequencies followed by the same letter are not significantly different ($P > 0{\cdot}05$).

comparing DNA-based results with those based on visual identification of wood decay fungi (Guglielmo *et al.* 2007, 2008b). This DNA-based assay is less expensive and time-consuming than other molecular techniques, such as methods based on RFLP or sequencing, thus representing an efficient diagnostic tool for tree inspection procedures (Nicolotti *et al.* 2010). This study was designed to determine an optimal sampling approach for standing trees to use in conjunction with the multiplex PCR assays, independent of the presence of obvious symptoms or signs of decay.

First, the sampling approach tested was based on an innovative way to collect wood samples by drilling holes into the bole of standing tree. The use of a 43-cm-long bit can provide samples as long as the wood cores commonly extracted using a Pressler increment borer. With respect to this latter device, a drilling-based sampling is faster and allows for the collection of sawdust, a substrate that makes for easier DNA extraction when compared to solid wood. To minimize injuries to sampled trees, we employed drill bits 4 mm in diameter because it has been shown that holes of comparable size can heal without the need of artificial plugging (Grissino-Mayer 2003).

Second, we chose to sample trees at the base of the trunk because in this location root and butt rot fungi can both be detected. Because these fungi are regarded as the most hazardous for tree stability, their detection in a tree demands further inspections focused on the assessment of the extent and severity of their decay. However, it should be noted that some wood decay fungi, i.e. *Inonotus hispidus* and *Inonotus obliquus*, just to name a few, are predominantly found above the base of the trunk and are frequently associated with stem fractures or cankers. If such fungi are the main targets of the survey or in presence of structural defects on the stem, it may be more appropriate to sample the trunk further up from the base.

Third, the number of drillings and the possibility to pool different samples before analysis were the two variables tested in this work. The reduction in the number of drillings per tree makes a sampling method less invasive. Similarly, the analysis of a unique pooled sample, rather than multiple samples derived from different drillings, markedly reduces costs and time of diagnosis.

Efficacy of each sampling scheme was determined by comparing data obtained through the combination of results of six drillings analysed independently (i.e. 100% diagnostic efficiency) with those of several less labour intensive and less invasive sampling schemes. According to our results, two out of the three approaches based on four drillings (4.2 and 4.4) were as effective as the six-drilling-based method. Conversely, assays based on one, two or three drillings were all significantly less sensitive than those obtained by four and six drillings analysed separately. Our results thus indicate that sampling intensity affected the overall diagnostic efficiency of wood decay fungi using the DNA-based assay described by Guglielmo *et al.* (2007, 2008b). On the other hand, pooling wood samples coming from the same tree did not significantly affect the results. In conclusion, scheme 4.2 undoubtedly provided the best results when considering both diagnostic efficiency and effort. Alternatively, in the case of large surveys, schemes 2.1 and especially 4.1 may be valid options. Although they are both less sensitive than the 4.2 and 4.4 approaches, the first scheme minimizes the number of drillings per tree and both schemes minimize the cost of analysis in the laboratory. In general, choice of the most appropriate method may vary depending on considerations such as acceptable injury levels, cost and the diagnostic sensitivity that is needed.

Note that prehomogenization of samples is time-consuming and does not significantly increase diagnostic efficiency of the assay; thus, it may not be worth performing.

Finally, results indicate diagnostic efficiency to be higher in smaller trees (DBH < 80 cm) when compared to that in larger trees (DBH > 80 cm). This information suggests that, when dealing with individual trees of large size, as in the case of monumental specimens, it may be appropriate to increase the number of drillings above four or six to increase the chances of identifying wood decay fungi. Similarly, in these situations, it may also be worth analysing each drilling separately to maximize diagnostic efficiency.

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