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Summary

Heterobasidion irregulare is one of the most destructive fungal pathogens of pines in North America and was accidentally introduced into central Italy, where it has become invasive. The fungus is currently recommended for regulation by the European and Mediterranean Plant Protection Organisation (EPPO). In this work, an efficient diagnostic tool for the early detection of *H. irregulare* based on Loop-mediated isothermal AMPlification (LAMP) coupled with two different DNA extraction methods was developed. The LAMP assay showed high specificity and good sensitivity, with a limit of detection of about 20 picograms of target DNA and time of detection of less than 40 minutes. The assay was successfully tested on a variety of different samples, including fungal fruiting bodies, infected plants, and colonized wood. A survey on environmental samples collected in the field was also performed by using the LAMP assay coupled with a rapid DNA extraction method. The possible applications of this molecular diagnostic tool encompass the monitoring of pine forests surrounding the current invasion area,

laboratory or in-field analyses of samples from suspected trees, and the surveillance in the ports of entry of wood imported from North America.

Introduction

The *Heterobasidion annosum* species complex includes two fungal species distributed in North America, i.e. *H. irregulare* Garbel. & Otrosina and *H. occidentale* Otrosina & Garbel., and three in Eurasia, i.e. *H. abietinum* Niemelä & Korhonen, *H. annosum* (Fr.) Bref. and *H. parviporum* Niemelä & Korhonen (Garbelotto & Gonthier, 2013). All species are necrotrophic pathogens regarded as some of the most destructive disease agents of conifers worldwide. Within the species complex, *H. irregulare* attacks pines (*Pinus* spp.) in North America (Otrosina & Garbelotto, 2010; Olson et al., 2012; Garbelotto & Gonthier, 2013). The disease can result in root rot, reduced growth, and mortality of host trees (Garbelotto & Gonthier, 2013). Primary infection is effected by basidiospores on freshly cut stumps or wounds, while secondary infection occurs by mycelium growth from stumps or diseased trees to healthy ones through root contacts (Garbelotto & Gonthier, 2013). The North American *H. irregulare* was accidentally introduced in central Italy during World War II (Gonthier et al., 2004), most likely through the movement of pallets or other military equipment made of untreated lumber from infected trees (Gonthier et al., 2004; Garbelotto et al., 2013). In the same area in central Italy, its Eurasian pine-associated sister species *H. annosum* is also present. The newfound sympatry between these two allopatrically diverged species favoured hybridization processes with unpredictable ecological consequences (Gonthier & Garbelotto, 2011). The introduction of *H. irregulare* has also resulted in an ongoing invasion process in coastal Italian stone pine (*P. pinea* L.) stands (Gonthier et al., 2007). The invasive species is seemingly outcompeting the native one, despite the two fungi displaying comparable pathogenicity levels towards pines

(Garbelotto et al., 2010). Indeed, *H. irregulare* can saprobically colonize a larger volume of wood and may produce a higher number of fruiting bodies than *H. annosum* (Giordano et al., 2014), two factors that may account for its invasiveness (Giordano et al., 2014; Gonthier et al., 2014). The relevant actual and potential impact of *H. irregulare* on European pine stands, the documented hybridization with the native *H. annosum*, and the ability to colonize oak stands (Gonthier & Garbelotto, 2011; Gonthier et al., 2012, 2014), have lead to the recent inclusion (September 2015) of the invasive pathogen in the A2 list of pests recommended for regulation by the European and Mediterranean Plant Protection Organisation (EPPO). As a consequence of this situation, efficient diagnostic tools are needed for the monitoring of this pathogen and for accurate and effective containment measures to be implemented in the previously defined zone of infestation (ZOI) and buffer zone (BZ) (Gonthier et al., 2014; EPPO, 2015).

Molecular methods are now widely and routinely used in plant disease diagnostics as they are generally efficient and sensitive, and they may allow early detection and identification of a range of plant pathogens and pests. Molecular diagnostic assays combined with appropriate sampling methods have been developed for the rapid detection of several wood decay fungi (Guglielmo et al., 2007, 2008, 2010, 2012; Gonthier et al., 2015). A tool recently developed by Lamarche et al. (2017) and based on qPCR TaqMan™ probes enabled the detection of *H. irregulare* in samples such as airborne particles captured by silicone-based spore traps. This tool offers a great example of how the molecular biology may be useful for plant pathological diagnostic purposes (Lamarche et al., 2017). Other molecular methods have been developed to discriminate different *Heterobasidion* species (Garbelotto et al., 1996; Gonthier et al., 2003) and to detect *Heterobasidion* spp. in wood samples (Gonthier et al., 2015), based on Taxon-Specific Competitive-Priming (TSCP) and Multiplex PCR, respectively. However, a reliable simple/fast/in-field assay for the detection of *H. irregulare* in environmental samples is still lacking.

PCR- and qPCR-based molecular methods are often time-consuming and require specialized operators able to perform and manage the laboratory analyses, as well as to interpret the results (Niessen et al., 2013). Moreover, they can rarely be applied in the field because of the requirement for large facilities and of the heterogeneous nature of environmental samples, most of which might contain PCR-inhibitor compounds. Loop-mediated isothermal AMPlification (LAMP) is an emerging molecular tool offering rapid, accurate, and cost-effective diagnosis of plant pathogens. In contrast to PCR-based methods, LAMP is an isothermal assay which does not require a thermocycler (Notomi et al., 2000). A typical LAMP assay uses two sets of primers (external primers F3 and B3, and internal primers FIP and BIP) and a DNA polymerase without exonuclease activity but with strand-displacing activity, in order to generate amplicons containing loop regions to which further primers (namely FL and BL) can bind (Notomi et al., 2000). As the enzyme for LAMP is more tolerant to chemical inhibitors often found in environmental samples compared to standard PCR polymerases, the LAMP assay enables the use of fast DNA extraction methods making it possible to perform diagnostics in the field (Tomlinson, 2013). Indeed, the application of LAMP-based assays has been described for the detection of a broad range of plant pathogens (reviewed in Ward & Harper, 2012), including several important fungal and bacterial tree pathogens (Tomlinson et al., 2007; Temple & Johnson, 2011; Bühlmann et al., 2013; Harrison et al., 2017), phytoplasmas (Tomlinson et al., 2010a) and wood-inhabiting nematodes (Kang et al., 2015).

Here, we report on the development of a LAMP diagnostic assay coupled with both a standard and a rapid DNA extraction method for the detection of *H. irregulare* in a variety of environmental samples, i.e. mycelia, fruiting bodies, plant samples and wood. The molecular diagnostic tool was developed following the EPPO Standards PM 7/98(2) guidelines (EPPO, 2014).

Materials and methods

Using available data from whole genome comparative analysis between *H. irregulare* and its sibling species *H. annosum* (Sillo et al., 2015), a candidate genomic locus for the design of the LAMP assay was selected on the basis of both strong intra-specific conservation and inter-specific divergence. Analytical specificity of primers was assessed by performing the LAMP assay on DNA extracted from the three taxonomically-related species within the genus *Heterobasidion* reported in Europe, i.e. *H. abietinum*, *H. annosum* and *H. parviporum*, and of other non-target wood decay fungi often associated with pines. Analytical sensitivity was assessed by performing the LAMP assay on independent dilution series of *H. irregulare* DNA. Selectivity, i.e. test of the matrix effects on the assay, was evaluated by performing the assay on different environmental-like matrices produced in the laboratory such as pine logs and seedlings artificially inoculated with the pathogen. A survey on environmental samples collected in the field and processed by using the rapid DNA extraction method was also performed. Finally, the assay was validated for repeatability and reproducibility.

Development of primers for Loop-mediated isothermal AMPlification (LAMP) assay

By using available BAM files of the whole genome comparative analysis between *H. irregulare* and *H. annosum* performed by Sillo et al. (2015), a *H. irregulare* genomic region was selected for the design of LAMP primers. *H. annosum* was indeed regarded as the most similar species to *H. irregulare* by several studies (Linzer et al., 2008; Dalman et al., 2010; Sillo et al., 2015). In detail, SamTools *mpileup* (Li et al., 2009) on BAM files was used to calculate nucleotide diversity along the aligned genomes to find a region showing high nucleotide diversity between *H. irregulare* and *H. annosum* and no intraspecific diversity within species. A small region (316 bp) showing high nucleotide diversity (> 20 Single Nucleotide Polymorphisms-SNPs/Kb) between species but no intraspecific nucleotide diversity (no SNPs among isolates of the same species) was used as a target region to design primers for LAMP (Figure 1). This genomic

region is located in the scaffold_03 inside a gene (Transcript ID: 472015) annotated as Cytochrome P450 monooxygenase with heme binding activity (Olson et al., 2012). The selected region showed 14 intra-specific conserved SNPs (Figure 1). Primers were designed on the *H. irregulare* sequence of the reference genome (Olson et al., 2012) using PrimerExplorer V.4 (https://primerexplorer.jp/e/) and standard parameter sets. The LAMP assay included six primers, namely external primers F3 and B3, internal primers FIP and BIP, and loop primers FL and BL. An additional set of primers developed by Tomlinson et al. (2010b) was used as internal control in the DNA extraction from plant materials, i.e. COX LAMP assay. Successful DNA extraction from plant samples was verified by scoring the positive signal in the COX LAMP assay. Primers were synthesized by Thermo Fisher Scientific and all sequences are shown in Table 1.

Fungal materials

In order to assess the sensitivity and specificity of the LAMP assay, mycelia from pure cultures were used (Table 2). Nine isolates of *H. irregulare* were selected as positive controls. Six out of nine were obtained from airspora and one from a fruiting body collected in the invasion area in central Italy and pre-screened for the absence of *H. annosum* introgressed alleles based on more than 500 independent AFLP loci (Gonthier & Garbelotto, 2011). Two out of nine originated in North America (Table 2). Six isolates of the native sibling species *H. annosum* were selected as non-target controls (closest non-target species in the genus *Heterobasidion*) (Table 2). In addition, three isolates of *H. abietinum* and three of *H. parviporum* were selected as additional non-target controls within the genus *Heterobasidion*. All isolates were previously identified through molecular diagnostic assays (Gonthier et al., 2003, 2007).

Seven other fungal taxa were selected as non-target controls since they are basidiomycetes often found in the same hosts as *H. irregulare*, i.e. pines: *Echinodontium tinctorium* (Ellis & Everh.) Ellis & Everh., *Fomitopsis pinicola* (Sw.) P. Karst., *Fuscoporia torulosa* (Pers.) T. Wagner &

M. Fisch., *Onnia* spp., *Phaeolus schweinitzii* (Fr.) Pat., *Porodaedalea pini* (Brot.) Murrill and *Stereum* spp. Two isolates were used for each of the above taxa (Table 2). Isolates were grown in 2% malt extract broth at 25°C for one week before being harvested. Approximately 200 mg of mycelium for each isolate was collected using a vacuum pump, freeze dried overnight and ground using four glass beads (two with diameter of 0.2 mm and two with diameter of 0.4 mm) in a FastPrepTM Cell Disrupter (FP220-Qbiogene). DNA extraction from mycelia was performed using EZNA® Stool DNA Kit (Omega Bio-Tek, USA) according to Gonthier et al. (2015).

Environmental samples

In order to test the selectivity, i.e. the variations of the sample materials/matrix (Boonham et al., 2016), three types of samples were used. The first included wood sawdust collected from logs of *Pinus sylvestris* L. artificially inoculated with *H. irregulare* and *H. annosum*; the second was *P. pinea* seedlings artificially inoculated with *H. irregulare* and *H. annosum*; the third comprised wood sawdust from drillings conducted at the base of standing *P. pinea* trees in disease centers in the invasion area of *H. irregulare*. The first two types of samples were prepared in the laboratory and are hereafter referred to as “environmental-like samples”, while the third is referred to as “environmental samples”.

In order to prepare samples of the first type, four 30 cm length - 20 cm diameter fresh *P. sylvestris* logs obtained from branches of healthy trees were inoculated with either *H. irregulare* (2 logs, isolate codes CP15 and 49SA) or *H. annosum* (2 logs, isolate codes Ha.carp. and CAL1) isolates in one of the two extremities as described by Giordano et al. (2014). One non-inoculated log was used as negative control. After eight weeks, each log was drilled four times as previously described (Guglielmo et al., 2010) to a depth of 10 cm and sawdust from drilling was collected for a total of 8 samples from logs inoculated with *H. irregulare*, 8 samples from logs inoculated with *H. annosum*, and 4 samples from the non-inoculated log (Table S1).

In order to obtain samples of the second type, 25 *P. pinea* seeds provided by Florsilva Ansaloni (Bologna, IT) were surface sterilized in 30% (w/v) hydrogen peroxide solution for 60 minutes with constant agitation, and rinsed twice with sterilized water. Subsequently, the embryos were detached and placed singly into 500 ml jars containing 50 ml of water-agar (15 g L^{-1} of agar). Jars were incubated under white light at room temperature (25±2°C). After 15 days, seedlings were inoculated with two isolates of *H. irregulare* (isolate codes 9OA and 48NB) and two of *H. annosum* (isolate codes 137OC and BM42NG) by placing two mycelial plugs of 6 mm by the pine stems (5 mm apart). For each fungal isolate, five replicates were prepared. Five pine seedlings not inoculated were included as negative controls. Seedlings were inspected daily for the presence of crown symptoms including needle discoloration, damping-off and death. As soon as the first symptoms appeared, stems were collected in 2-ml tubes and stored at -20°C (Table S1).

In order to obtain samples of the third type, 10 standing *P. pinea* trees showing crown symptoms and 10 fallen pine trees located in the "Gallinara pine plantation" (Anzio, Rome, IT), and 10 standing pine trees located into the gardens of the historical "Villa Doria Pamphjili" (Rome, IT), were sampled by drilling a single hole at the base of the trunk, as described in Guglielmo et al. (2010) for a total of 30 samples (Table S1). After each sampling, the drill bit was cleaned in 0.5% (w/v) sodium hypochlorite solution and washed with sterilized water. Four *Heterobasidion* fruiting bodies were also collected from fallen *P. pinea* trees in the "Circeo National Park" (Latina, IT) and included in the study as samples.

Standard and rapid DNA extraction

Two types of DNA extraction methods were used on all samples, i.e. a standard and a rapid method. The standard DNA extraction method was performed using the EZNA® Stool DNA Kit, as described by Gonthier et al. (2015). Before using the standard DNA extraction method, all samples were lyophilized overnight.

The rapid DNA extraction method was based on the use of alkaline polyethylene glycol (PEG) (Chomczynski & Rymaszewski, 2006). Briefly, samples (approx. 200 mg) were homogenized as a crude macerate using a 10-mm stainless steel bead in 5-ml plastic tubes containing 2 ml alkaline PEG lysis buffer (50 g L^{-1} of PEG average Mn 4,600, 20 mM KOH, pH 13.5). Tubes were shaken by hand for 2 minutes, and 1 µl of the ten-fold dilution of the crude macerate was used in the LAMP assay.

LAMP conditions

The reaction mixes for the LAMP assay included 200 nM of the external primers F3 and B3, 500 nM of the internal primers FIP and BIP, 500 nM of the loop primers FL and BL, 15 µl of Isothermal Mastermix ISO-001 (OptiGene, UK) and 1 µl of DNA or crude macerate extract. LAMP reactions were carried out using a CFX Connect Real-Time PCR detection system (Bio-Rad, USA) equipped with FAM reading channel. The protocol was 65°C for 40 minutes with read plate every minute. The programme for the calculation of melting curves was: ramp from 65°C to 95°C with a temperature increment of 0.1°C and a read plate every 10 seconds. Amplification and melting curves were analyzed using the Biorad-CFX manager software (Bio-Rad, USA).

Analytical specificity and sensitivity assays

Analytical specificity was assessed on 9 isolates of *H. irregulare* (target species), 12 isolates of other *Heterobasidion* spp. (non-target species most closely related in the genus), and 14 isolates of fungal species often found in the same host as *H. irregulare* (Table 2). DNA concentration of non-target samples was estimated with a Nanodrop (Thermo Scientific, USA) and only concentrations over 10 ng μl^{-1} were used in the specificity assay. Two technical replicates were performed for each biological replicate.

Analytical sensitivity was assessed by performing the LAMP assay on three independent tenfold dilution series (ranging from 30 ng μl^{-1} to 30 10^{-7} ng μl^{-1}) of DNA of three *H. irregulare* isolates (isolate codes 49SA, 91NA and 48NB), tested in triplicate. DNA concentrations were estimated by using a Nanodrop and 5 µl of fungal DNA were diluted on 45 µl of extract of *P. pinea* DNA collected from a pine seedling, in order to mimic natural conditions. The Limit of Detection (LOD) was calculated by multiplying the one-sided t-distribution value (degree of freedom = 6) *versus* the standard deviation among 7 samples (DNA from isolates 9OA, 1116-1, Conk1, 48NB, 38NA, CP15 and 49SA; see Table 2) diluted until the observed limit of detection in the analytical sensitivity test (Hibbert & Godding, 2005).

Selectivity and validation of rapid DNA extraction method and LAMP assay on environmental samples

The selectivity assay was performed on 75 different samples (see "environmental samples" section). Two technical replicates were performed for each biological replicate. Validation of the assay for samples of DNA extracted using the standard method was performed by comparing the outcomes of the LAMP assay to those obtained by using standard molecular techniques to detect *Heterobasidion* spp. In detail, PCR with taxon-specific primers for *Heterobasidion* spp. was performed (Gonthier et al., 2015), amplicons sequenced and compared with other sequences in GenBank using the blastn algorithm.

This type of validation was not possible for samples of DNA extracted using the rapid method, since no DNA was amplifiable by PCR. Therefore, for these samples validation was performed by comparing the outcomes of PCR with taxon-specific primers on samples extracted using the standard method.

Diagnostic sensitivity (*DSe*), or true positive rate, and specificity (*DSp*), or true negative rate, were calculated as follows:

$$1)\ DSe = \frac{\Sigma TP}{\Sigma TP + FN} \quad 2)\ DSp = \frac{\Sigma TN}{\Sigma TN + FP}$$

where *TP* are True Positive values (positive samples in the standard assay), *TN* are True Negative values (negative samples in both standard and LAMP assays), *FN* are False Negative values (negative samples in LAMP assay only), and *FP* are False Positive values (positive samples in LAMP assay only) (Altman & Bland, 1994). The Positive Likehood Ratio (LR+) was calculated as the ratio between *DSe* and the false positive rate, while Negative Likehood Ratio (LR-) was calculated as the ratio between the false negative rate and *DSp*. Calculation of the likelihood ratios and their 95% confidence intervals was performed with the internet online Diagnostic Test Calculator (available at: http://araw.mede.uic.edu/cgi-bin/testcalc.pl). Repeatability and reproducibility were assessed by performing twice the standard DNA extraction and LAMP assay and the rapid DNA extraction and LAMP assay by two different operators. Five positive and five negative DNA samples were used in each assay.

Results

Analytical specificity and sensitivity assays

Using the LAMP assay coupled with the standard DNA extraction method on fungal isolates, no amplification was observed in negative controls. The LAMP assay developed for *H. irregulare* did not show cross-amplification with other *Heterobasidion* species, including *H. abietinum*, *H. annosum* and *H. parviporum*, nor with seven other wood decay-causing Hymenomycetes often found in pine trees (Figure 2).

Detection of fluorescence in LAMP reactions performed with a tenfold serial dilution of positive controls (*H. irregulare* DNA) resulted in a time of detection ranging from 6 to 27 minutes, depending on the starting DNA concentration (Figure 3). Optimum results in term of

time of amplification were obtained with DNA concentrations of approximately 3 ng μl^{-1}. Increased dilutions substantially affected the time of detection, and no amplification before 40 minutes was observed for dilutions over 3 x 10^{-3} ng μl^{-1}. Based on the results of the LAMP assay on lower concentrations of seven positive controls (seven different concentrations of approx. 0.03 ng μl^{-1}, with standard deviation = 0.00538), the minimum LOD of the assay was 1.99 x 10^{-2} ng μl^{-1} (19.9 pg μl^{-1}).

Selectivity and validation of the rapid DNA extraction method and LAMP assay on environmental samples

All 45 environmental-like samples showed a positive signal in the COX LAMP assay, confirming the effectiveness of both DNA extraction methods (Figure 4). Neither false positive nor false negative results were obtained by using the LAMP assay coupled with the standard DNA extraction method on environmental-like samples, confirming the robustness of the assay (Table 3 and Table S1). The value of *Dsp* was 1.0 (100%) and the value of *Dse* was 1.0 (100%). Likelihood ratios LR+ and LR- considering 40% of positive samples were both infinite.

One false negative result was obtained when the LAMP assay was performed on DNA extracted with the rapid extraction method (Table 3 and Table S1). As a consequence, for these samples only LR+ was infinite, while the LR- was close to 0 (0.06) (Figure 5). The *Dse* decreased to 0.964 (96.4%).

On a total of 30 environmental samples collected in the field, five samples were positive for *Heterobasidion* spp. after taxon-specific PCR. Sequencing of amplicons confirmed that one out of five was positive for *H. irregulare*. Only the sample containing *H. irregulare* DNA was positive to the LAMP assay, and with both DNA extraction methods (Table 4 and Table S1). This sample was collected from a fallen *P. pinea* tree in the “Gallinara pine plantation” (Table S1). Three out of four fruiting bodies collected at the “Circeo National Park” were positive for *H. irregulare* after the LAMP assay coupled both DNA extraction methods. Repeatability and

reproducibility of the LAMP assay were confirmed by the outcomes of the analyses performed by two different operators. In the repeatability and reproducibility assays, there were no significant differences in terms of time of detection of the pathogen when analyses were performed by different operators (*t test*; p-value < 0.05).

Discussion

This work successfully developed a rapid and specific diagnostic LAMP assay for the forest pathogen *H. irregulare*. The availability of the genome of *H. irregulare* (Olson et al., 2012) and the comparative genomic analysis between this fungus and its sister species *H. annosum* (Sillo et al., 2015), which is closely related from a genomic perspective, have allowed the selection of an appropriate species-specific locus to design the primer set for the LAMP assay. The approach we used to select this locus is a good example of how data from whole genomic studies have practical applications in forest pathology. The recent improvements of sequencing techniques favoured a rise in numbers of sequenced and released fungal genomes, as successfully demonstrated by the Fungal Genomics Program (FGP; http://genome.jgi.doe.gov/programs/fungi/index.jsf) launched by the Joint Genome Institute (JGI) of the US Department of Energy (Grigoriev et al., 2011). In the near future, fungal genomics is expected to further support plant pathologists involved in the development of molecular diagnostic tools.

The LAMP assay developed in this work showed high specificity and sensitivity in the detection of *H. irregulare* DNA from different sample materials, including mycelia and fruiting bodies, pine seedlings and wood samples. The trustworthiness of the molecular tool was assessed using 35 fungal isolates and 75 between environmental-like and environmental samples. All *H. irregulare* DNA samples were successfully amplified by using the LAMP assay, showing clear and consistent positive results. The use of a Real-Time PCR detection system coupled with

OptiGene reagents allowed the assay to be completed in less than 40 minutes. The minimum amount of target DNA detected by the LAMP assay (19.9 pg μl^{-1}) was comparable to that observed in other assays for forest pathogens including *Phytophthora ramorum* Werres, De Cock & Man in 't Veld (10 pg μl^{-1}; Tomlinson et al., 2007), *Phytophthora kernoviae* Brasier, Beales & S.A. Kirk (17 pg μl^{-1}; Tomlinson et al., 2010b) and *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (7 pg μl^{-1}; Harrison et al., 2017). Real time PCR assays for *H. irregulare* (Lamarche et al., 2017) showed a higher sensitivity than the molecular assay developed here, however both primer set and TaqMan™ probe were designed on multi-copy genomic regions, i.e. the Internal Transcribed Spacer (ITS). In our study, the development of two different primer sets for LAMP designed on ITS regions failed to specifically amplify *H. irregulare* and showed cross-amplification with DNA of *H. annosum* (data not shown). The genic region selected to design primers for the LAMP assay is deemed to be present as a single copy. The differences in the two LOD values between Real Time PCR and LAMP assays can thus be regarded as a consequence of the different copy number of target regions as previously suggested (Chern et al., 2011).

The LAMP assay was validated perfectly on sample DNA extracted with the standard method. In fact, for these samples LR+ and LR- were infinite. An infinite likelihood ratio indicates that if the test is positive/negative, the pathogen is definitely present/absent.

A DNA sample extracted with the rapid extraction method from a log inoculated with *H. irregulare* showed a false negative result to the LAMP assay, which affected the values of LR- and of *Dse*. Taken together, these findings suggest that there is a small probability that an infected sample will prove negative in the assay coupled with the rapid DNA extraction method. However, it should be noted that the other three samples coming from the same log as the one showing the false negative result were positive in the LAMP assay. Therefore, the false negative result is likely to be due to the very low quantity of fungal materials in the sample. It is worth noting that that the diagnostic efficiency of molecular analysis on wood samples is strongly

affected by sampling (Guglielmo et al., 2010). A sampling scheme based on four drillings significantly increased the efficiency of the diagnostic test compared to samplings based on single drillings in standing trees (Guglielmo et al., 2010). An optimal sampling procedure for the LAMP assay coupled with the rapid extraction method could include at least four samples from different drillings *per* tree, which can be further pooled together as a single sample, as previously suggested (Guglielmo et al., 2010). The rapid extraction method can be considered a practical alternative to the standard DNA extraction method we tested since it does not require specific equipment or expensive reagents. In addition, it is much less time consuming compared to the standard method (2 minutes of sample homogenization *versus* ~ 40-60 minutes of DNA extraction with commercial kits), favouring application in the field.

The successful development of a LAMP assay combined with a rapid DNA extraction method and working on a broad range of sample materials is expected to reduce the time currently needed to perform routine diagnostics with other methods, and could pave the way to application of this tool for in-field diagnosis. In fact, even though the LAMP assay was developed and validated on a Real Time PCR detection system, it can be performed using in-field portable equipment, e.g. Genie® II from OptiGene, as documented in comparable studies (Lenarčič et al., 2013).

In the Pest Risk Analysis (PRA) for *H. irregulare*, EPPO argued that the spread of this pathogen would potentially have a considerable impact on European pines in the long term (EPPO, 2015). Practical solutions for surveillance and monitoring of this pathogen will be pivotal not only in Italy, but in all European countries where pines or other hosts are present. Applications of the LAMP assay encompass the periodic monitoring of pine trees in the buffer zone surrounding the current invasion area, with emphasis on laboratory or in-field analyses of samples from suspected trees, and surveillance in the ports of entry of wood of hosts imported from North America. In addition, this rapid diagnostic tool may be useful in North America, where *H. irregulare* stands among the most harmful forest pathogens (Garbelotto & Gonthier, 2013).

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Figure legends

Fig. 1. Genomic alignment between *H. irregulare* and *H. annosum* of the selected locus for the design of the LAMP primer sets. Black bars represent the locations of the designed primers (F3, B3). Forward primer FIP includes F1c (complementary) and F2 regions. Backward primer BIP includes B1c (complementary) and B2 regions. Polymorphic sites between the two species are highlighted in light grey.

Fig. 2. Results of the analytical specificity assay. (a) Amplification curve of positive control (*H. irregulare*) and no amplification of non-target controls (DNA from *H. abietinum*, *H. annosum*, *H. parviporum*, and other seven fungal species found in pines) as detected by the CFX Connect Real-Time PCR detection system. (b) Melting curves displayed as negative first derivative of the fluorescence-*versus*-temperature plot over the temperature (-d(RFU)/dT *versus* T). For a better resolution of the image, only one representative replicate for each species is visualized.

Fig. 3. Results of the analytical sensitivity assay. (a) Amplification curve of different dilutions of a positive control (*H. irregulare* isolate 48NB) as detected by the CFX Connect Real-Time PCR detection system. (b) Jitter plot of the three independent dilution series. The quantity of DNA (ng; x-axys) is plotted against the detection threshold time of the instrument (minutes).

Fig. 4. Example of sample (wood sawdust from an inoculated *P. sylvestris* log – sample ID LOG1-CP15/b) positive to COX and *H. irregulare* LAMP assays. (a) Amplification plot of plant DNA as detected by the COX LAMP assay (grey line) and of *H. irregulare* DNA as detected by the specific assay (black line). (b) Melting curves displayed as negative first

derivative of the fluorescence-*versus*-temperature plot over the temperature (-d(RFU)/dT *versus* T). NC: Negative control.

Fig. 5. Nomogram for likelihood ratios of the LAMP assay coupled with the rapid DNA extraction method considering a hypothetical pre-test probability of presence of *H. irregulare* of 40% (as expected for environmental-like samples). Blue line represents LR+, while red line represents LR-.

Fig. 1

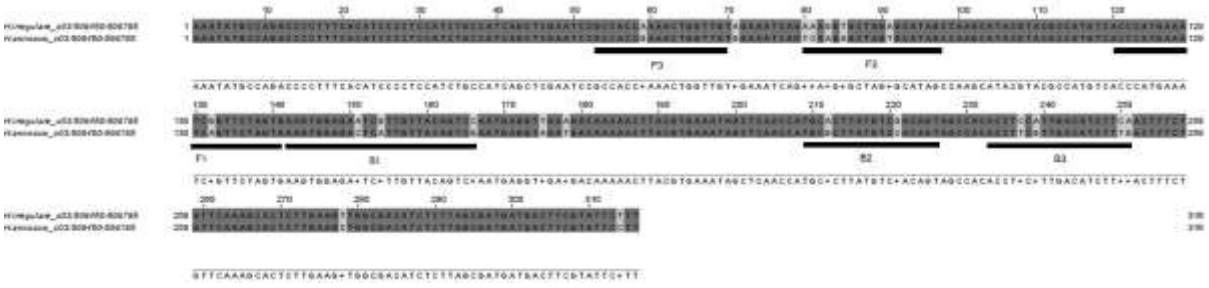

Fig. 2

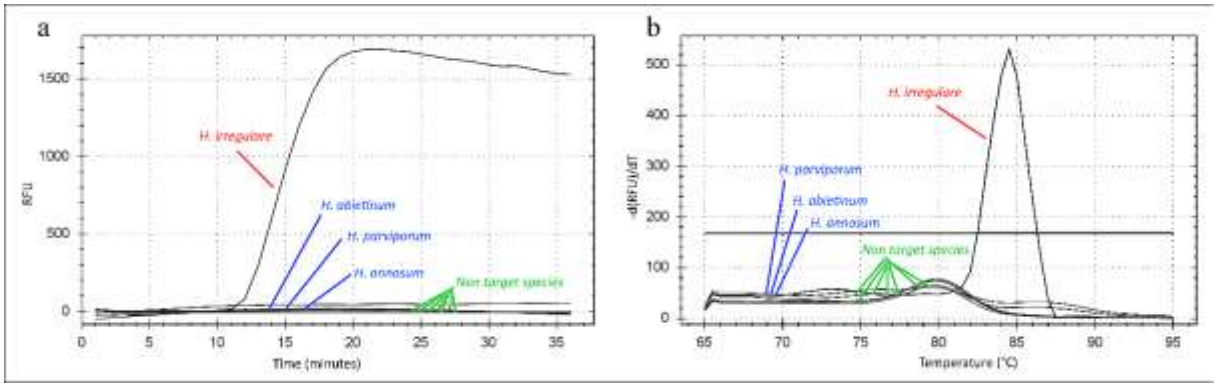

Fig. 3

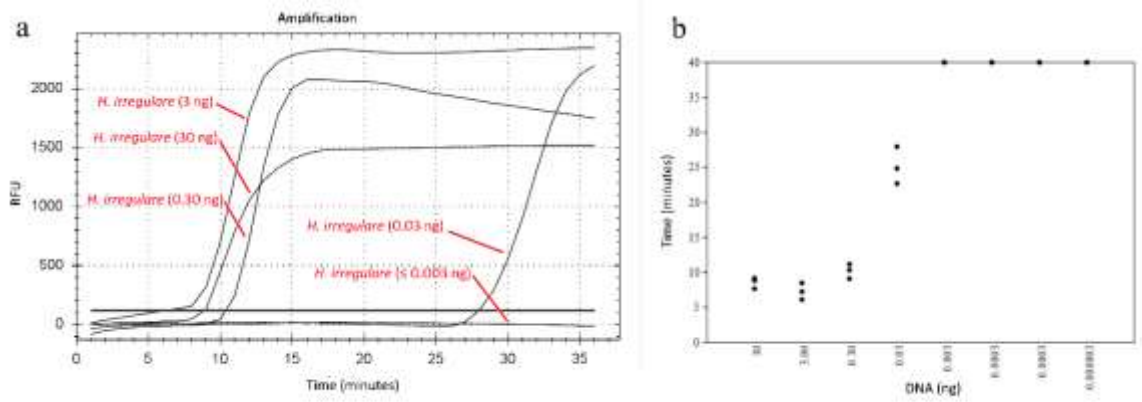

Fig. 4

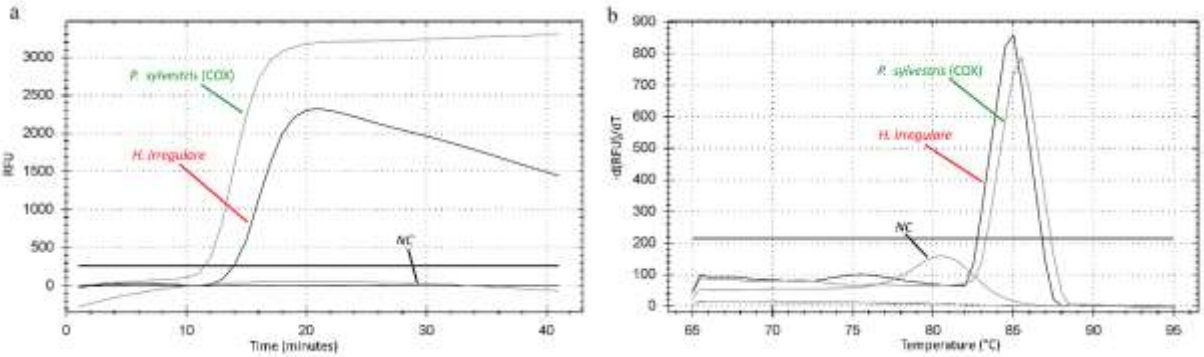
a
RFU
P. sylvestris (COX)
H. irregulare
NC
Time (minutes)
b
-dRFU/dT
P. sylvestris (COX)
H. irregulare
NC
Temperature (°C)

Fig. 5

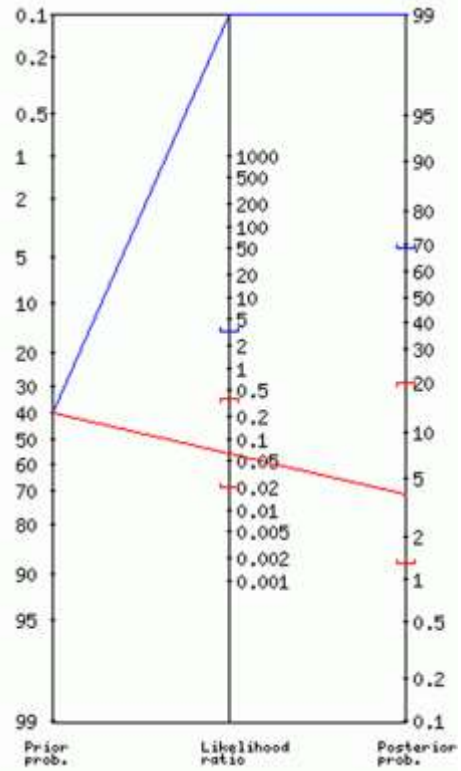
Prior prob.
Likelihood ratio
Posterior prob.

Table 1. Primers used in the LAMP assay.

Primer name	**Sequence (5′–3′)**	**Source**
HirrSC3_F3	GCCACCAAAACTGGTTGT	This study
HirrSC3_B3	TGAAGATGTCAATGGAGGT	This study
HirrSC3_FIP	TCACTAGAACCGATTTCATGGGTAAAGGTGCTAGAGCATAGC	This study
HirrSC3_BIP	AGTGGAGAATCGTTGTTACAGTCCACTGTCGACATAAGTGCA	This study
HirrSC3_FL	ACATGGCGTACGTATGCTTG	This study
HirrSC3_BL	GAGGTTGAAGACAAAAACTTACGTG	This study
COX-F3	TATGGGAGCCGTTTTTGC	Tomlinson et al., 2010b
COX-B3	AACTGCTAAGRGCATTCC	Tomlinson et al., 2010b
COX-FIP	ATGGATTTGRCCTAAAGTTTCAGGGCAGGATTTCACTATTGGGT	Tomlinson et al., 2010b
COX-BIP	TGCATTTCTTAGGGCTTTCGGATCCRGCGTAAGCATCTG	Tomlinson et al., 2010b
COX-FL	ATGTCCGACCAAAGATTTTACC	Tomlinson et al., 2010b
COX-BL	GTATGCCACGTCGCATTCC	Tomlinson et al., 2010b

Table 2. List of fungal isolates used in this study.

Species	**Isolate ID code**	**Host/Source-Geographic origin**	**Source or MUT[1] Accession number**	**Use in the assay**
Heterobasidion irregulare	9OA	airspora-Italy	MUT00003629	**Positive samples for the target species**
Heterobasidion irregulare	39NE	airspora-Italy	MUT00001193	
Heterobasidion irregulare	48NB	airspora-Italy	MUT00003627	
Heterobasidion irregulare	38NA	airspora-Italy	MUT00001161	
Heterobasidion irregulare	49SA	airspora-Italy	MUT00003628	
Heterobasidion irregulare	91NA	airspora-Italy	DISAFA[2]	
Heterobasidion irregulare	CP15	*Pinus pinea*-Italy	MUT00003560	
Heterobasidion irregulare	1116-1	*Pinus taeda*-USA	ESPM	
Heterobasidion irregulare	Conk1	*Pinus taeda*-USA	ESPM	
Heterobasidion abietinum	38EF	airspora-Italy	DISAFA[2]	**Non-target samples morphologically and taxonomically related**
Heterobasidion abietinum	P137r	airspora-Italy	MUT00005618	
Heterobasidion abietinum	VPS	*Pinus strobus*-Italy	MUT00005577	
Heterobasidion annosum s.s.	BM42NG	airspora-Italy	MUT00003543	
Heterobasidion annosum s.s.	137OC	airspora-Italy	MUT00003656	
Heterobasidion annosum s.s.	109SA	airspora-Italy	MUT00003538	
Heterobasidion annosum s.s.	CAL1	*Fagus* sp.-Italy	MUT00001215	

Heterobasidion annosum s.s.	Ha.carp.	*Pinus pinea*-Italy	MUT00001143	
Heterobasidion annosum s.s.	Sib2	*Pinus sylvestris*-Russia	MUT00005583	
Heterobasidion parviporum	A2B	airspora-Italy	DISAFA[2]	
Heterobasidion parviporum	P162r	airspora-Italy	MUT00005615	
Heterobasidion parviporum	Cep7	*Picea abies*-Italy	DISAFA[2]	
Echinodontium tinctorium	Aho-60-88-R	N/A-USA	ESPM	**Non-target samples often found in the host plant**
Echinodontium tinctorium	FP-47304-T	N/A-USA	ESPM	
Fomitopsis pinicola	C-Joux	*Picea abies*-Italy	MUT00005674	
Fomitopsis pinicola	FFP2	*Abies alba*-Italy	DISAFA[2]	
Fuscoporia torulosa	759	N/A-Czech Republic	CCBAS	
Fuscoporia torulosa	DP39	*Prunus pissardi*-Italy	MUT00005649	
Onnia leporina	Phaeo1	*Pinus sylvestris*-Italy	DISAFA[2]	
Onnia tomentosa	OT-Slu	*Picea abies*-Sweden	SLU	
Phaeolus schweinitzii	CeMiCa	*Cedrus* sp.-Italy	DISAFA[2]	
Phaeolus schweinitzii	574	*Picea abies*-Italy	MUT00005653	
Porodaedalea pini	14	*Pinus halepensis*-Italy	BPV	
Porodaedalea pini	28	*Pinus halepensis*-Italy	BPV	
Stereum hirsutum	27E.3	*Castanea sativa*-Italy	MUT00005631	
Stereum sanguinolentum	12	*Larix decidua*-Italy	MUT00005673	

[1] Mycotheca Universitatis Taurinensis

[2] only DNA extract available

BPV, Department of Biology and Plant Pathology of the University of Bari (Italy); CCBAS, Department of Experimental Mycology, Institute of Microbiology, Czechoslovak Academic of Sciences, Videnska (Prague, Czech Republic); DISAFA, Department of Agricultural, Forest and Food Sciences of the University of Torino (Italy); ESPM, Department of Environmental Science, Policy and Management of the University of California in Berkeley (USA); SLU, Swedish University of Agricultural Sciences (Sweden); N/A, data not available.