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ARTICLE

Molecular techniques for pathogen identification and fungus detection in the environment

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Abstract: Many species of fungi can cause disease in plants, animals and humans. Accurate and robust detection and quantification of fungi is essential for diagnosis, modeling and surveillance. Also direct detection of fungi enables a deeper understanding of natural microbial communities, particularly as a great many fungi are difficult or impossible to cultivate. In the last decade, effective amplification platforms, probe development and various quantitative PCR technologies have revolutionized research on fungal detection and identification. Examples of the latest technology in fungal detection and differentiation are discussed here.

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INTRODUCTION

Fungi represent the greatest eukaryotic diversity on earth and they are among the primary decomposers in ecosystems. It is conservatively estimated that 1.5 million species of fungi exist (Hawksworth 1991). Many fungal species are important plant and human pathogens (Agrios 2005). Rapid and accurate detection of fungal pathogens to species or strain level is often essential for disease surveillance and implementing a disease management strategy. Developing direct detection assays is challenging because fungal pathogens can exist as multiple species complexes or at very low concentration in clinical and natural environments. Different molecular genotypes/varieties can also exist within species, and may have different pathogenic profiles and virulence levels to the host. In addition, unculturable and non-sporulating fungi remain a major challenge when studying biotrophic, endophytic, and mycorrhizal groups. Therefore, novel techniques are required when attempting to detect fungi in the environment.

Increasingly molecular techniques are employed in studies requiring the detection of fungi in the environment. In this paper, based on presentations at a Special Interest Group meeting convened during the International Mycological Congress (IMC9) in Edinburgh, UK, August 2010, some of the latest diagnostic techniques employed in the detection of fungi, including fluorescence *in situ* hybridisation (FISH), DNA array technology, Multiplex tandem PCR, and Padlock probe technology with rolling circle amplification and loop-mediated isothermal amplification (LAMP) were discussed. The importance of DNA extraction and sampling methodologies were also briefly discussed.

GENERAL CONSIDERATIONS

Suitable methodologies to isolate nucleic acids from the enormous range of habitats that fungi can occupy are crucial to all molecular detection methods. Critical to the successful isolation of nucleic acids are the methods used for extraction and sample preparation, as well as the sampling strategy employed.

DNA extraction methods

An enormous variety of nucleic acid extraction methods is available. For many applications, commercial kits are available, but these are not always suitable. For example, in the case of soil, none of the kits presently available are able to extract efficiently using sample sizes typically required (see below). Therefore, with unusual and difficult sample types, customized methods are typically employed. Key to this is the cell disruption/homogenisation step in the presence of an appropriate buffer (typically a CTAB or guanidinium thiocyanate based buffer). A wide variety of methods can be used, including specialist equipments such as a planetary ball mill (Brierley *et al.* 2009), appropriate bead beaters, pressure cycling technology (Okubara *et al.* 2007), and novel approaches using equipment such as a paint shaker for soil samples (Reeleder *et al.* 2003), or even a conventional food blender for plant and food samples. All cell disruption steps are potentially damaging to the nucleic acid quantity and quality, either through direct shearing of the nucleic acid, or through the co-extraction of humic acids (an inhibitor of PCR) present in plant and soil samples. Therefore, methods which provide an adequate level of cell disruption to enable satisfactory nucleic acid extraction, but without too much nucleic acid damage or release of potential PCR inhibitors, are required.

Following cell disruption and subsequent extraction steps, the nucleic acid can be purified using standard methods, including pelleting, silica membrane spin filter, and silica-magnetic particle separation. When selecting a purification step, consideration needs to be given to the nucleic acid quality/quantity required, cost, speed of extraction and ability to automate any process. The method of analysing the nucleic acid will determine what purification method is used. For example, DNA profiling and metagenomic studies using next generation sequencing approaches will require pure, high quality nucleic acid extracts. Conversely, cruder, less pure extracts may be used where real-time PCR is used for routine diagnostic purposes where the emphasis is on nucleic acid quantity, speed and cost of extraction because the downstream application is more tolerant of impurities in the DNA extract.

Sample preparation

Innovative sample preparation can enhance chances of detection. For example, organic matter can be removed from soil and processed separately for fungal targets which survive solely in that component of the soil. Sclerotium-forming pathogens may be separated from the rest of the soil by sieving or floating prior to nucleic acid extraction. These approaches effectively concentrate the fungal target, thereby increasing the chance of successful detection. Where appropriate, a baiting approach could be used in conjunction with nucleic acid extraction and PCR. This has the added benefit of confirming the target is viable.

Sampling strategy

In addition, an appropriate and statistically robust sampling strategy should be utilised. This will vary between fungal targets, depending upon the biology (and possibly epidemiology) of the organism to be detected. For example, a 'W' sampling strategy across an area may not be appropriate for a target with a highly clustered distribution, and a grid sampling approach should be used instead. The individual sample size processed for DNA extraction should also be large enough to be biologically relevant (Ophel-Keller *et al.* 2008). A wide variety of methods exist, and no one approach is likely to be suitable for all targets. Knowledge of the biology of the target species is essential for designing and determining the optimum sampling and extraction methodology in any particular case.

SEQUENCING INDEPENDENT METHODS

DNA sequencing of the internal transcribed spacer (ITS) and large subunit (LSU) regions of rRNA, followed by comparative sequence analysis, has been the 'gold standard' for molecular identification of most fungi, particularly of culturable fungi. This strategy is fast and accurate, but is dependent on sequence quality in existing reference databases. In many recently evolved fungal groups, however, the ribosomal genes are insufficiently variable, and sequencing of additional genes is necessary. Costs could also be a challenge for routine diagnostic laboratories, and therefore various sequencing independent methods that are available can be used.

Fluorescent *in situ* hybridisation

Since fungi are ubiquitous, it is important to understand their biodiversity and abundance, as well as their ecological roles in different habitats, such as soil, decaying leaves and wood, and also indoor environments or humans. Indeed, it is critical to identify the metabolically active species ('real players') in communities or ecosystems in order to understand the ecosystem processes that involve fungi. PCR based methods, such as fingerprinting or molecular cloning, do not discriminate between 'real players' and fungi that are dormant. Also, DNA and RNA extraction protocols may be biased due to rigid cell walls of fungal hyphae. Immuno-staining provides a means to identify species *in situ* but is extremely laborious in the experimental preparation. All these kinds of technical difficulties, can, however, be overcome by fluorescence *in situ* hybridisation (FISH).

FISH is a powerful method for the *in situ* detection of active growing organisms in environmental samples (Amann *et al.* 1995). The technique can visualize the precise location

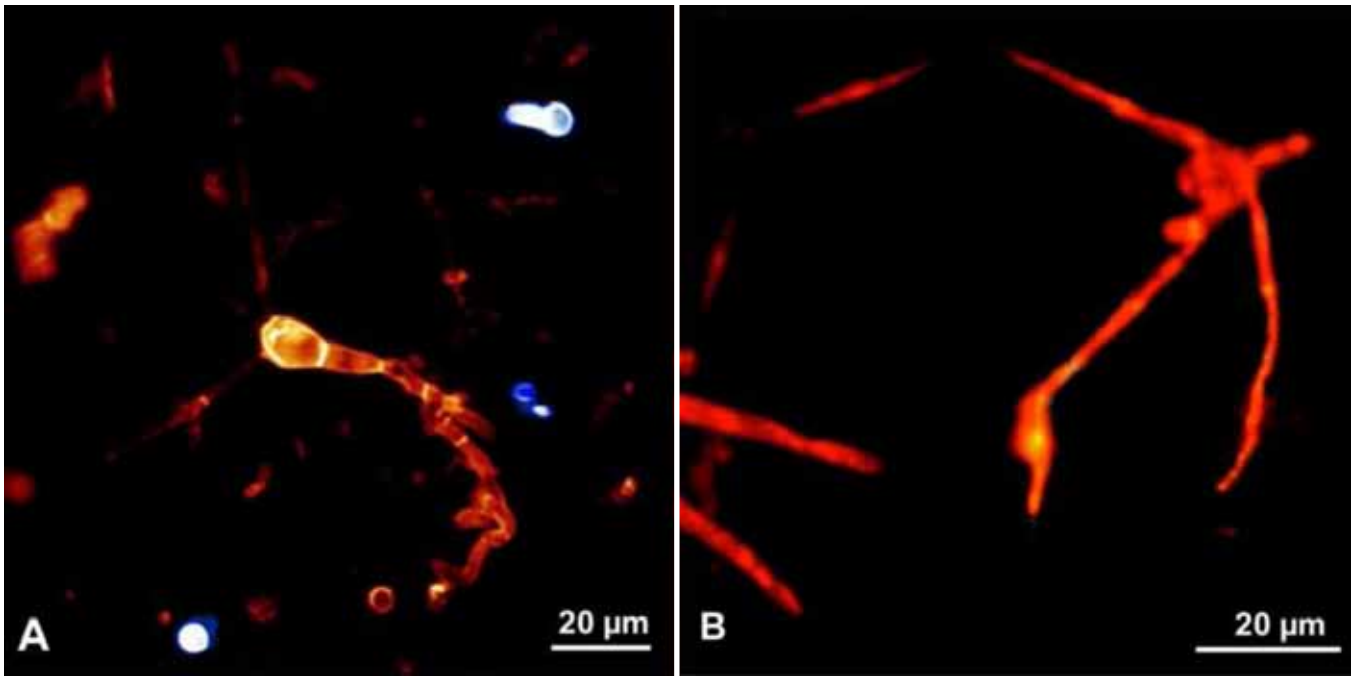

Fig. 1. Examples of fluorescence *in situ* hybridisation. **A.** *Clavariopsis aquatica* growing on aquatic leaf litter, probed with 18 rRNA-targeted MY1574 domain specific probe (from Baschien *et al*. 2008). **B.** Accessibility of *Tetracladium marchalianum* conidia for FISH 28SrRNA-targeted species specific probe TmarchB10 (modified from Baschien *et al.* 2008).

of particular DNA or RNA sequences in the cytoplasm, organelles, or nuclei of biological materials. As a result, the technique can detect metabolically active fungi directly in the environment without cultivation when RNA is present. The spatial distribution of growing mycelia on or within colonized substrata can also be investigated (Li *et al*. 1996, Baschien *et al*. 2001, McArthur *et al*. 2001, Robin *et al.* 1986).

The major step of FISH involves the preparation of biological materials or environmental samples, and the labelling (incorporation of a fluorescent label/marker) of a nucleic acid sequence to form a probe. Then, under controlled experimental conditions, the probe is hybridized to the DNA or RNA in biological materials to form a double-stranded molecule. Finally, the sites of hybridization are detected and visualized. FISH is commonly used in the 'top-bottom approach' research followed with feedback to the top level (Amann *et al*. 1995). The molecular biology and genetics of targeted organism is well-understood, and the probe is designed based on the nucleotide sequence which has been well characterized.

FISH probes often target sequences of ribosomal RNA or mitochondrial genes because they are abundant in sequence databases and in multiple copies in each cell. The probes can be designed by computer-assisted search from target organisms for "signatures". These signature regions can be specific at different phylogenetic levels depending on the variability of the target molecule sequences. The probe comprises a short sequence, ranging from 15 to 20 nucleotides, that is specific for one or several taxa at species, genus, or higher taxonomic ranks. The probe or oligonucleotide is then labelled with a fluorochrome, for example a carboindocyanine dye (CY3).

A particular attraction is that FISH probes can be applied to formaldehyde or ethanol fixed environmental samples, or to cultures. At the ribosomes, the probe will specifically anneal to its complementary sequence resulting in a heteroduplex. After incubation, a washing step is crucial to discriminate the target from non-target organisms. Probes that do not bind to an rRNA sequence will be washed away, resulting in a fluorescent signal of exclusively target organisms. The probe conferred signal is correlated with the ribosome content, and therefore increases in cells with higher metabolic activity.

The first FISH probe targeting a living fungus was designed by Li *et al.* (1996) for *Aureobasidium pullulans* on the phylloplane of apple seedlings; this was the first time that a living fungus had been visualized by FISH. Examples of situations in which the method has been used are very varied. Fungi belonging to *Eurotiomycetes* were demonstrated to be more abundant than *Dothideomycetes* in an extremely acidic mine drainage using 18S rRNA-targeted probes (Baker *et al*. 2004). Baschien *et al.* (2008) designed nine taxon-specific probes targeting the 18S or 28S rRNA for the detection of aquatic hyphomycetes in leaf litter (Fig. 1A, B). Domain specific probes were also used to detect active fungi in mice (Scupham *et al*. 2006) and in waste water sewage granule biofilms (Weber *et al*. 2007). Most probes have been designed to target the 18S or 28S rRNA gene, and their specificity needs to be kept under review as sequence databases expand.

Several factors can influence the efficiency of FISH, for example the sterical and electrostatical properties of rRNA, the features of the probe, and hybridisation conditions such as the fixation method, buffers, the stringency of probe binding, and incubation time. Inacio *et al.* (2003) investigated

the different accessibility of the rRNA molecule for FISH probes. They designed 32 different probes that targeted the 26S (large subunit) of rRNA for yeasts, and investigated the conferred signal intensity generated from the probes. They concluded that the prerequisite for successful FISH is the specificity of probes (Inácio *et al*. 2003).

Limitations of the FISH method can include fungal and substrate inherent autofluorescence, insufficient permeability of cell walls, non-specific binding of probes, and low ribosome contents. Autofluorescence of fungi can also lead to false positive fluorescence signals. A fluorescence scan conducted by confocal laser scanning microscopy revealed that many freshwater fungi emit a green autofluorescence (Baschien *et al*. 2001), and, consequently, a green-labelled FISH probe could not be used. It is, therefore, important to check for autofluorescence of both target and non-target organisms before selecting the fluorochrome for probe labelling. Environmental samples and substrates such as leaves, wood, and animal tissues, also emit strong autofluorescence in several wavelengths at the same time, interfering with probe signals. However, the pin-hole technique of a laser scanning microscope is helpful in reducing background emissions compared to conventional epifluorescence (Baschien *et al*. 2001).

Low or no permeability of fungal cell walls can lead to weak or no signals because of the failure of FISH probes to penetrate rigid cell walls (Brul *et al*. 1997). One possible way to overcome this problem is to use cell wall lysing enzymes, such as chitinases and glucanases. A more elegant method is the use of peptide nucleic acid (PNA) probes. PNA probes are mimics in which the negatively charged sugar-backbone of DNA is replaced with a non-charged polyamide backbone. PNA probes share the same nucleotide bases as the conventional DNA probes. However, PNA probes penetrate cell walls more effectively due to their neutrality and they do not have to overcome the destabilizing electrostatic repulsion during hybridisation (Wilson *et al*. 2005, Shepard *et al*. 2008).

Low ribosome contents can arise from either a scarcity of potential substrate in oligotrophic habitats, or the presence of components inhibiting fungal growth. However, the Catalysed Reporter Deposition FISH (CARD-FISH) is a variant of the FISH method designed to enhance probe conferred signals. The major difference is that the probes are labelled with horse-radish-oxidase instead of a fluorochrome. Fluorescence-labelled tyramide is then added to the cells after hybridisation. The horse-radish-oxidase catalyses the deposition of the reporter, and this reaction leads to 20 to 30 fold stronger FISH signals than conventional FISH. Consequently, the number of active rRNA molecules necessary to detect a probe conferred signal decreases by using CARD FISH (Pernthaler *et al*. 2002).

DNA array hybridization

DNA array hybridization, also known as Reverse Dot Blot Hybridization (RDBH) or macroarray, is a technique based on hybridization of amplified and labelled genome regions of interest to immobilized oligonucleotides spotted on a solid support platform. It was originally developed to detect mutations of human genes, and is still an important diagnostic tool in clinical research (Chehab & Wall 1992, Yang *et al*. 2001, Zhang *et al*. 1991). It is now considered a powerful and practical technique for the detection and identification of fungi and other microbes, such as bacteria, from complex environmental samples without the need for isolation in culture (Chen *et al*., 2009, Ehrmann *et al*. 1994, Lévesque *et al*. 1998, Tambong *et al*. 2006, Uehara *et al*. 1999, Wu *et al*. 2007, Zhang *et al*. 2007, 2008). For this type of application, oligonucleotides, or microcodes (Summerbell *et al*. 2005), are designed from a taxonomically complete dataset of suitable genome region(s) (Chen *et al*. 2009, Tambong *et al*. 2006). The oligonucleotides can be selected manually, by analysing multi-sequence alignments, or through computer programs, such as SigOli (Zahariev *et al*. 2009) and Array Designer (Premier Biosoft International, Palo Alto, CA). Synthesized oligonucleotides with 5'-amine modifications are then spotted onto a supporting platform, such as a nylon membrane or glass slide, either manually or robotically. Robotic spotting can significantly increase the maximum density of the array which can favour the detection of broader taxonomic groups (Chen *et al*. 2009). Amplicons of the target gene region(s) are amplified by PCR, labelled with digoxygenin (DIG) and subjected to the DNA hybridization procedure previously described (Fessehaie *et al*. 2003). A positive reaction between an amplicon and a perfectly matched (PM) oligonucleotide generates a chemiluminescent signal which can be detected by X-ray film or a digital camera in dark rooms. Captured images are then analysed on a computer program such as GenePix Pro (Molecular Devices, Sunnyvale, CA).

The design of species or group-specific oligonucleotides is a crucial step in this process since it defines the specificity and sensitivity of the assay (Lievens *et al*. 2006, Pozhitkov *et al*. 2006, Urakawa *et al*. 2003). It is generally agreed that the length of the oligonucleotide, the number, type and position of SNPs contained in an oligonucleotide, determine its discriminatory potential for DNA hybridization. A desirable oligonucleotide should: (1) have suitable thermodynamic characters such as melting temperature; (2) contain as many polymorphic sites as possible located close to the centre or on the 3'-half of the sequence; and (3) have the least probability of hairpin or dimer formation (Kawasaki *et al*. 1993, Lievens *et al*. 2006). Oligonucleotide lengths ranging from 25 to 35 mer have displayed the best balance between specificity and sensitivity (Chen *et al*. 2009). Longer lengths (> 35 mer) of oligonucleotides reduce the ratio of mismatched to matched base pairs, yet increase the number of bases available for hybridization, providing lower specificity but higher sensitivity to an array (Dorris *et al*. 2003, Lievens *et al*. 2006). Oligonucleotides up to 70 mer, however, have been used in macroarrays for the detection of plant viruses using gene sequences that are widely different between the closely related pathogens (Agindotan & Perry 2007, 2008). It has also been demonstrated that more complicated parameters, such as actual sequence arrangement and the mismatched duplex type, and their interactions, can play important roles

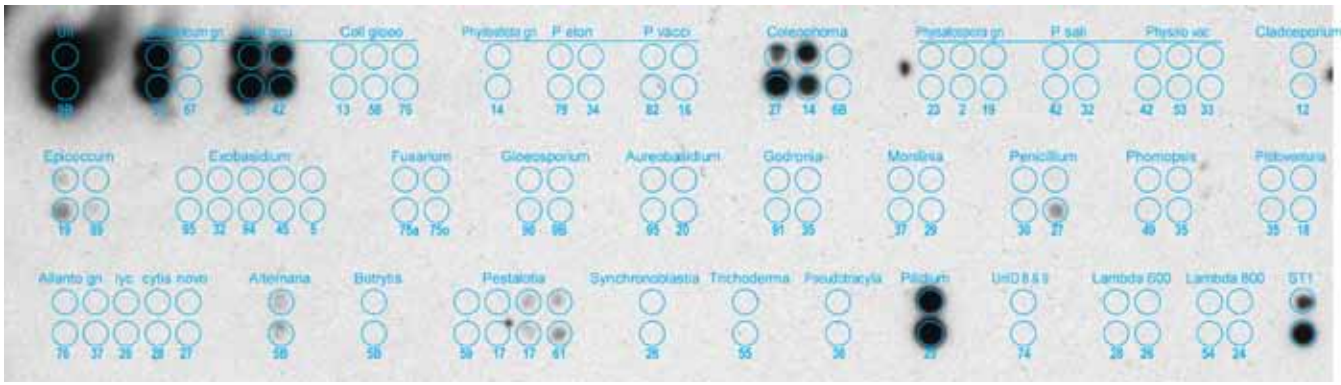

Fig. 2. DNA macroarray hybridization results from frozen cranberry fruit sample collected from Massachusetts, USA (adapted from Robideau *et al*. 2008). The x-ray film is overlaid with the oligonucleotide spotting pattern on DNA array membrane for cranberry fruit rot fungi. The top left pair of spots and bottom right pair of spots are oligonucleotides which served as positive controls. This array is showing positive signal for *Phyllosticta*, *Coleophoma*, *Epicoccum*, *Godronia*, *Alternaria*, *Pestalotia*, and *Pilidium*.

in affecting the discriminatory power of an oligonucleotide (Urakawa *et al*. 2003, Lievens *et al*. 2006, Pozhitkov *et al*. 2006). Despite numerous attempts to predict the behaviour of oligonucleotides in DNA hybridization, the thermodynamic interaction between the probes and oligonucleotides remains poorly understood (Pozhitkov *et al*. 2007).

Most DNA macroarrays that have been developed are based on a single region for the detection of a specific taxonomic group. Among these regions, 16S ribosomal DNA has been used for the detection of bacteria (Fessehaie *et al*. 2003, Xiong *et al.* 2006). Various genome regions, such as ribosomal DNA spacers (ITS), mitochondrial genes (e.g. cytochrome oxydase c subunit 1, *cox*1) and some protein coding regions (β-tubulin, EF-1α, etc), were chosen to target fungi and fungus-like organisms (Chen *et al.* 2009, Gilbert *et al*. 2008, Harper *et al*. 2011, Ning *et al*. 2007, 2008, Seifert & Lévesque 2004). Oligonucleotides with higher specificity are often designed from polymorphic sites located at indels present in multi-sequence alignments (Robideau *et al*. 2008, Tambong *et al*. 2006). Oligonucleotides extracted from a locus that can be well aligned with low sequence divergence tends to cross-react with non-target amplicons and display strong false positive signals even when tested with pure cultures because of the low frequency of polymorphisms in the specific oligonucleotides. This has been observed in arrays using *cox*1 in *Penicillium* subgen. *Penicillium* (Chen *et al*. 2009, Seifert *et al*. 2007). In a recent study, DNA arrays were constructed from multiple loci of *Phytophthora* species, including *ITS*, *cox*1 and the intergenic region (*cox*2-1 spacer, *CS*) between cytochrome c oxidase 2 (*cox*2) and *cox*1 (W. Chen *et al*. pers. comm.). In comparison to the *cox*1 region, the length variation and the presence of indels in the sequence alignments of *ITS* and *CS* provided better opportunities to select highly specific oligonucleotides. The combination of all three arrays increased the discrimination potential, detection accuracy, and redundancy of the assay.

DNA arrays have been developed for the detection of plant pathogens in a wide range of environmental samples, such as greenhouse crops (Le Floch *et al*. 2007, Lievens *et al.* 2003), potatoes (Fessehaie *et al*. 2003), ginseng (Punja *et al*. 2007), and fruits (Robideau *et al*. 2008, Sholberg *et al*. 2005, 2006). Macroarrays are also effective diagnostic tools for the detection of phytopathogenic bacteria (Fessehaie *et al*. 2003), fungi and fungus-like organisms (Chen *et al*. 2009, Lévesque *et al*. 1998, Tambong *et al*. 2006), nematodes (Uehara *et al*. 1999), and viruses (Agindotan & Perry 2007, 2008).

DNA array hybridization is highly sensitive as are most PCR-based approaches. With the unlimited capacity for the accommodation of oligonucleotides on one membrane and the reusability of the membranes, it shows superior multiplexing detection capability at a lower cost over other PCR-based methods. In a biodiversity study, the species profile could be revealed by hybridizing the oligonucleotide array with a mixed pool of DIG-labelled PCR products amplified from the total DNA of a query sample. This assay would be especially valuable for the simultaneous detection of multiple plant pathogens which cover a broad taxonomic range but are specific to a certain host. As an example, Robideau *et al*. (2008) developed a DNA array for cranberry fruit rot (CFR) fungal pathogens with over 2000 field samples being tested. This array had the ability to detect species from 24 genera of fungi known on cranberry with a single test. Fig. 2 shows that this DNA macroarray was able to detect species of *Phyllosticta*, *Cladosporium*, *Epicoccum*, *Godronia*, *Alternaria*, *Pestalotia*, and *Pilidium* from a single frozen cranberry fruit sample (Robideau *et al*. 2008).

The macroarray technology is now available commercially in four European countries under the name DNA Multiscan (http://www.dnamultiscan.com) and at the Guelph Pest Diagnostic Clinic (http://www.guelphlabservices.com/) for the test of plant pathogens in greenhouse nutrient solutions and roots of crops. There are very significant advances in next generation sequencing technology that have reduced the need of DNA array technology in functional genomics. However, there are developments in lab-on-a-chip array technology for very quick and for large-scale testing. A recent example is a low density array for *Phytophthora* detection, where amino-

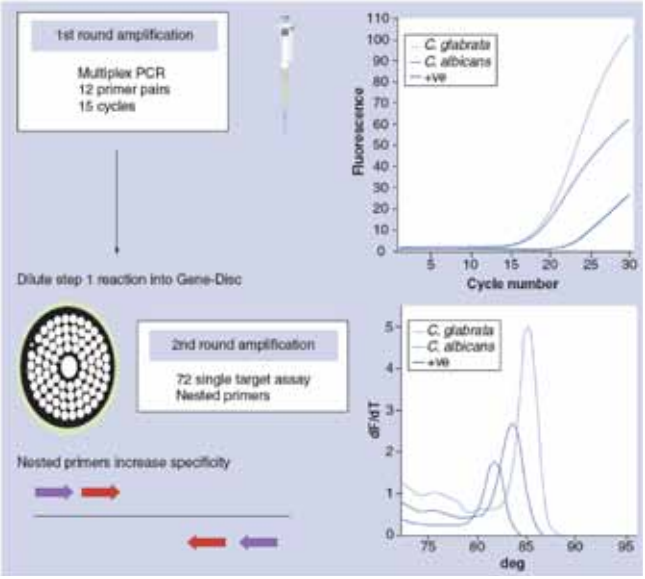

Fig. 3. Schematic representation of the multiplex tandem–PCR procedure illustrating specific detection and identification from a blood sample containing *Candida albicans* and *C. glabrata*. Reproduced from Lau *et al*. (2009) with the permission of Future Medicine.

labelled oligonucleotides are spotted over a gap between two electrodes inside a microchip (Julich *et al*. 2011). Hybridization is detected by a current passing through a silver deposit over the gap. Because of such developments, array-based assays are likely to remain a popular option for diagnostics.

Multiplex tandem PCR

Multiplexed-tandem PCR (MT-PCR) is a technology platform developed for highly multiplexed gene expression profiling and the rapid identification of clinically important pathogens (Stanley *et al*. 2005). The platform consists of two rounds of amplification (Fig. 3). In the first step, a multiplex PCR is performed at 10 to 15 cycles to allow enrichment of target DNA without creating competition between amplicons (Lau *et al*. 2009). This product is diluted and used as template for the second amplification that consists of multiple individual quantitative PCR reactions with primers nested within those used in the multiplex PCR. Up to 72 different PCR reactions can be multiplexed and performed simultaneously. Fluorescence is measured by SYBR green technology at the end of each extension cycle, and melt-curve analysis provides species-specific or gene-specific identification. The incorporation of two sets of species-specific primers for each target ensures correct amplification and detection, thus avoiding the expense of DNA probes. SYBR green detection also increases the multiplexing and quantitative capacity of real-time PCR systems, which are usually limited by the availability of fluorescent channels and the need to optimize each individual multiplex PCR.

In the clinical setting, invasive fungal infections (IFIs) remain a leading cause of morbidity and mortality in immune-compromised hosts. MT-PCR was considered a suitable alternative for the rapid detection and identification of fungal pathogens directly from clinical specimens, thus circumventing the need for gold standard culture and histology, which is slow, insensitive and non-specific. Faster and accurate molecular identification would also enable better guidance and earlier administration of targeted antifungal therapies. As such, the laboratory at Westmead hospital, in conjunction with AusDiagnostics (Alexandria, NSW, Australia), developed several MT-PCR assays to detect the16 major causes of fungal bloodstream infections. The targets included 11 species of *Candida*, *Cryptococcus neoformans* complex, *Saccharomyces cerevisiae,* pan-*Fusarium, F. solani,* and *Scedosporium prolificans*. Primers were designed using sequence variations within the ITS regions, elongation factor 1-α (EF1-α), and β-tubulin genes. Fungal targets were selected according to their frequency of causing infections, their potential resistance to frontline antifungal agents (especially fluconazole), and their high attributable mortality.

The MT-PCR platform was initially evaluated on 70 blood cultures in which a yeast or mould was seen in Gram's stain preparations, as well as 200 bacterial blood cultures and 30 samples which did not flag positive (Lau *et al*. 2008a). The sensitivity and specificity of the assay was 100 %. This included the correct identification of fungi in five cases with bacterial co-infection. In addition, no interference was observed in simulated cases of polyfungal infection. Unfortunately, three rare disease-causing yeasts (*Candida lambica, C. nivariensis* and *Kodamaea ohmeri*) were not detected by MT-PCR as targets were not available on the fungal panel. Nevertheless, this study demonstrated the diagnostic usefulness of the platform to rapidly identify common fungal pathogens within four hours of blood culture flagging (including automated nucleic acid extraction), which is considerably faster than the 48-96 h required by gold standard methods.

An expanded version of the MT-PCR fungal platform was then evaluated on 255 EDTA whole blood, 29 serum, and 24 plasma specimens obtained from 109 patients with proven candidemia (Lau *et al*. 2010) with the aim of circumventing the technical and sensitivity issues inherent in routine blood culture diagnosis. Although the MT-PCR assay was less sensitive than blood culture (75% sensitivity), the diagnosis of candidemia and pathogen identification was expedited by up to four days. The results also supported the contention that serum and plasma samples were better than whole blood samples for the molecular detection of candidemia.

Using a technique known as colony MT-PCR (Lau *et al*. 2008b), the fungal assay was also able to provide rapid (1.5 h) and unambiguous identification of yeasts direct from primary isolation plates without the need for DNA extraction or separation from mixed fungal/bacterial cultures. As such, colony MT-PCR offered a faster and better alternative to biochemical assays which are often subjective, prone to misidentification, and dependent on a pure culture being obtained. Current work is aimed at integrating identification panels with targets to detect molecular mechanisms of antifungal resistance.

Overall, the fungal MT-PCR assay compares favourably to other commercial platforms and integrates well into the

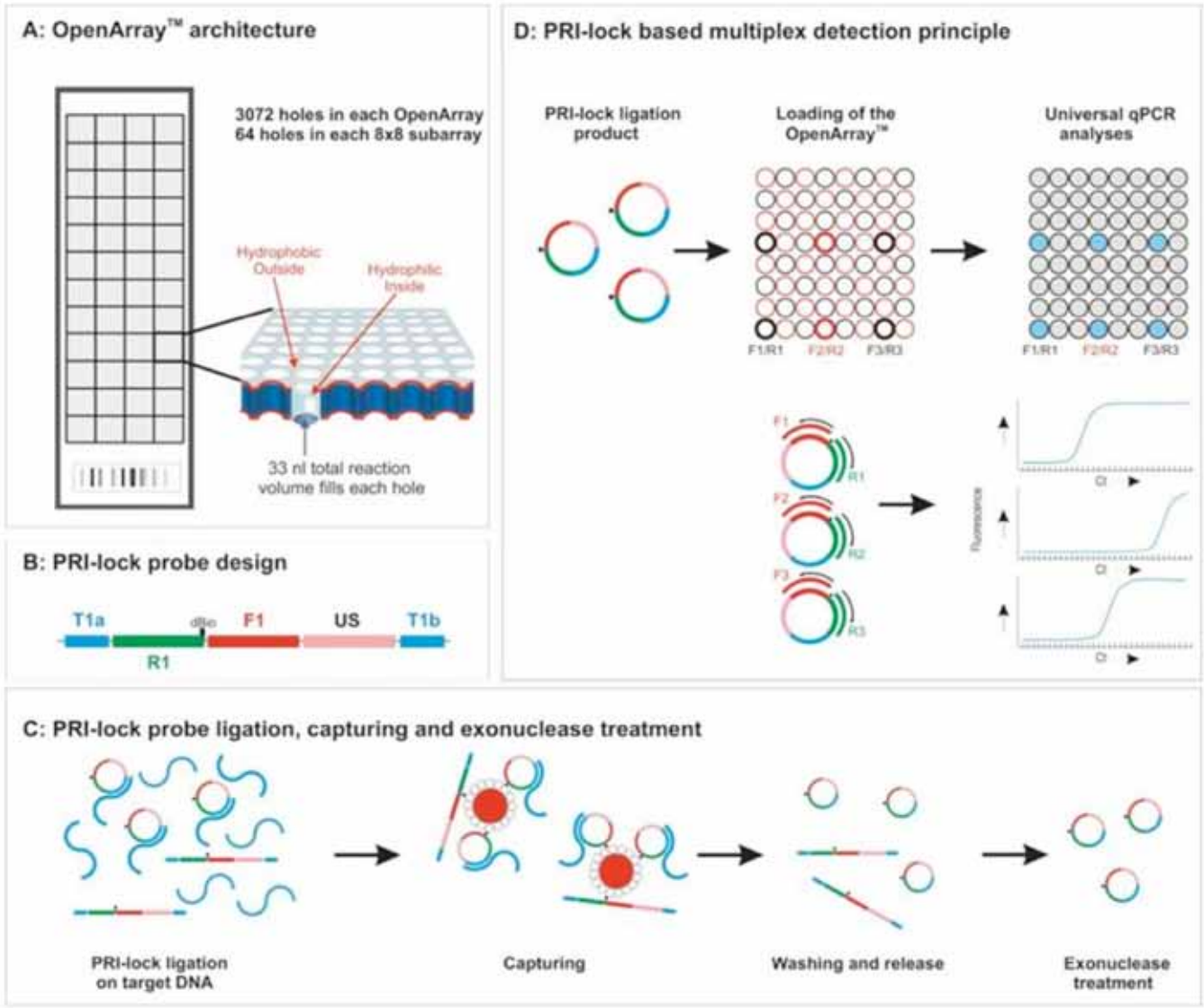

Fig. 4. Schematic overview of the padlock principle combined with OpenArray®, Technology for multiplex detection of three different targets (adapted from van Doorn *et al*. 2007).

routine workflow of diagnostic laboratories. Automated operations and use of commercial reagents further enables standardized procedures to be established. Nevertheless, the major limitation of all molecular diagnostic assays is target availability, which is often dictated by an effort to balance out costs and turn-around time with maximum throughput. The increased sensitivity of molecular assays and its ability to detect viable and non-viable cells should also place heavy emphasis on interpreting results with other microbiological data and clinical information.

ISOTHERMAL SYSTEMS

Isothermal systems do not require a thermal cycler to produce rapid temperature changes, but require only a simple platform such as heating blocks or water bath. Isothermal systems include rolling circle amplification (RCA) and loop-mediated isothermal amplification (LAMP).

Padlock probe technology and rolling circle amplification

Detection and characterization of single nucleotide polymorphisms (SNPs) is becoming increasing popular for pathogen identification, but was considered a major challenge for conventional real-time PCR using regular oligonucleotides detected by fluorescent dyes (e.g. SYBR green or TaqMan probes). In order to recognize SNPs among different genotypes, padlock probe techniques are required. Padlock probes (PLPs) are long oligonucleotides (about 100 bp) carrying a non-target-complementary segment flanked by the target complementary regions at their 5' and 3' ends, which recognize adjacent sequences on the target DNA (Nilsson *et al*. 1994). Thus, on hybridization, the ends of the probes occupy adjacent positions, and can be joined by enzymatic ligation (Figs 4–5). Ligation occurs and the probes are circularized only when both end segments correctly recognize the target sequences (Landegren *et al*. 1988). The helical nature of double-stranded DNA (dsDNA) enables the

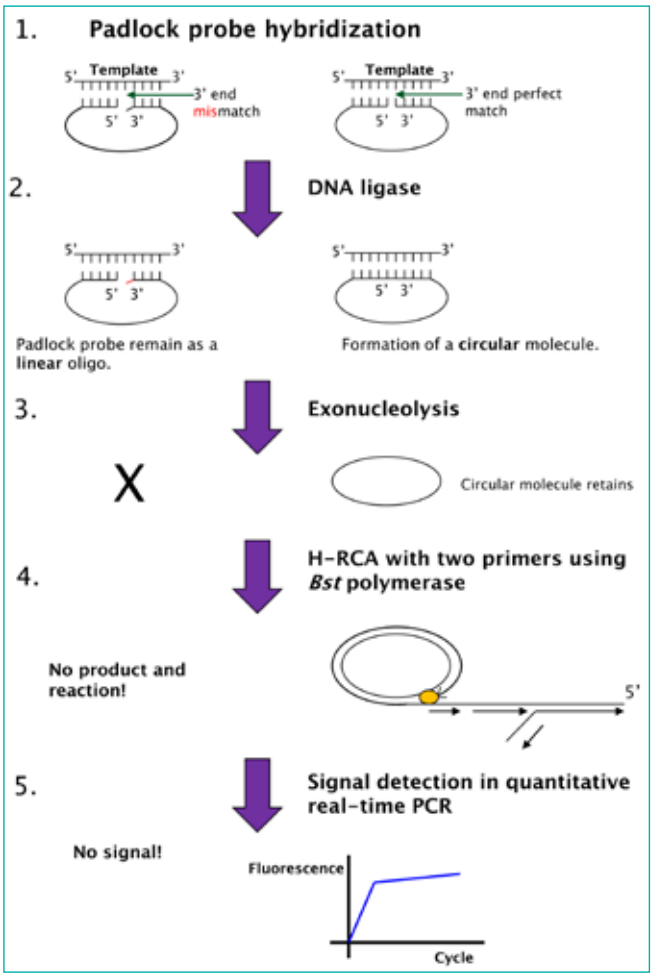

Fig. 5. Schematic representation of the steps in padlock probe technology coupled with hyperbranched rolling circle amplification (H-RCA) for SNPs detection. **1.** The hybridization of padlock probes (containing the complementary sequences at the 5' and 3' ends) to the target templates. **2.** During a perfect match, the probe forms a circular molecular with the aid of DNA ligase; while in the case of mismatch, no circular molecules formed. **3.** Non-hybridized template will be removed during the exonucleolysis reactions (digestion by exonucleases I and III). **4.** H-RCA is performed using two pre-designed primers and *Bst* polymerase, but no amplification will take place in the absence of a circular molecular. **5.** The accumulation of dsDNA products during isothermal rolling circle amplification of DNA minicircles is monitored in a real time PCR thermocycler with the addition of SYBR green.

probe to topologically bind to the target strand and the probe can't be displaced (Nilsson *et al*. 1994).

Padlock probes were initially introduced for *in situ* DNA localization and detection (Nilsson *et al*. 1994). They were developed originally for discrimination of centromeric sequence variation in human chromosomes (Nilsson *et al*. 1997). However, the method has now been applied to detection of genetically modified organisms (Prins *et al*. 2008). This concept also provides extensive multiplex potential for pathogen detection as the interaction between padlock probes does not give rise to circular molecules, which can be easily removed from the detection system (Nilsson *et al*. 1994, 1996). Recently, padlock probe-based applications for multiplex quantitative targets detection and for genotyping fungal and microbial community analysis using high throughput real-time PCR on OpenArrays® have been developed (van Doorn *et al*. 2007). Advantages of PLP-based diagnostic applications developed are a flexible and easily adaptable design, specificity, and multiplexing, universal downstream processing after ligation, and high-throughput format with real-time analysis (Tsui *et al.* 2012) (Fig. 4).

Briefly, various padlock probes are designed to target organisms and ligated to DNA extracted from environmental samples or cultures (van Doorn *et al*. 2007). Targets for ligation present in complex DNA samples such as soil or re-circulated water can be generated by PCR pre-amplification, through *Phi29* polymerase and whole genome amplification to ensure efficient detection. Real-time quantification for multiple targets is performed in OpenArrays®, which can accommodate up to 3072 x 33 nl PCR amplification with preloaded probe-specific primers (Fig. 4). Multiplex padlock ligation is followed by single-plex amplification using unique probe-specific primer pairs and SYBR green based detection in nano-litre PCR array (van Doorn *et al*. 2007). The performance of the padlock probe detection system has been demonstrated using 13 probes targeting several plant pathogens at various taxonomic levels (Szemes *et al*. 2005, van Doorn *et al*. 2007). All probes specifically detected their corresponding targets, and provided perfect discrimination against non-target organisms with very similar target sites. Pathogen quantification was robust in single target *versus* mixed target assays. This novel assay enables very specific, high-throughput, quantitative detection of multiple pathogens over a wide range of target concentrations, and should be easily adaptable for versatile diagnostic purposes (van Doorn *et al*. 2007).

Also, padlock probes containing zip-code sequence or a biotin-labelled moiety and internal endonuclease cleavage site, in conjunction with quantitative PCR and Luminex™ technology or microarray technology, can be used for multiplex pathogen detection and quantification (Szemes *et al*. 2005, Eriksson *et al*. 2009, van Doorn *et al.* 2009).

Alternatively, the signal by which the target hybridizes perfectly to the padlock probes can be amplified by hyperbranched rolling circle amplification (H-RCA) (Banér *et al*. 1998). Rolling circle amplification (RCA) is based on the rolling replication of short single stranded DNA (ssDNA) circular molecules (Lizardi *et al*. 1998, Fire & Xu 1995). RCA involves a single forward primer complementary to the linker region of the padlock probe and a DNA polymerase with strand displacement activity in an isothermal condition (Pickering *et al*. 2002). As a result, the padlock probe signal can be amplified several thousand-fold because the polymerase extends the bound primer along the padlock probes for many cycles and displaces upstream sequences, producing a long ssDNA molecule comprising multiple repeats of the probe sequence. Two primers are employed: a first forward primer that binds to the padlock probe and initializes RCA, and a

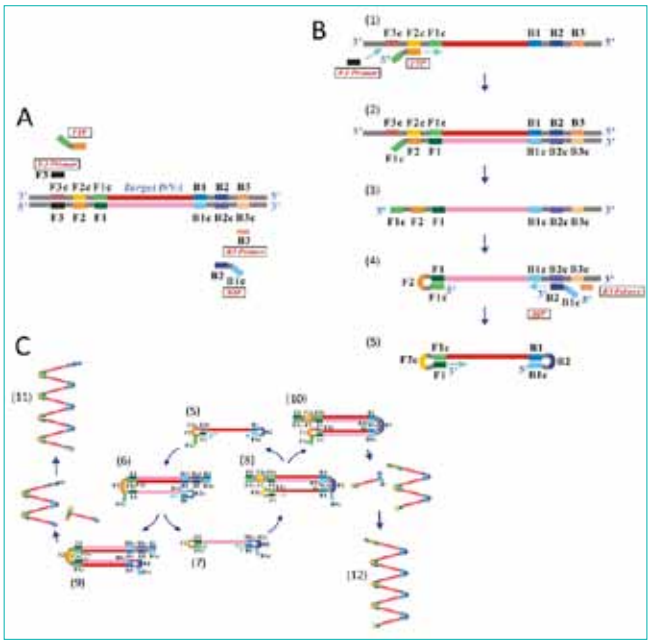

Fig. 6. Schematic representation of the mechanism of LAMP. **A.** General location of the LAMP primer set in relation to defined regions of the target DNA. Forward (F3) and backward (B3) outer primers and forward (FIP) and backward (BIP) inner primers are indicated. **B, C.** Basic principle and amplification steps in LAMP. In general new DNA strands are synthesizes from the F3 and B3 primers, and these strands are recognized by FIP and BIP to start loop-mediated autocycling amplifications. The final products are stem-loop DNAs with several inverted repeats of the target DNA, and cauliflower-like structures bearing multiple loops (modified from diagrams at <http://loopamp.eiken.co.jp/e/lamp/principle.html> © Eiken Chemical.

second primer that targets the repeated ssDNA sequence of the primary RCA product, finally generating large numbers of copies of the DNA fragments. This is called hyperbranching RCA (H-RCA) (Lizardi *et al*. 1998) (Fig. 5).

Padlock probe coupled with H-RCA offers a significant advantage for the detection of SNPs (Tsui *et al.* 2012). The formation of circular probes *via* ligation occurs when both ends of the padlock probes perfectly hybridize to the target at juxtaposition (Fig. 5). The subsequent H-RCA amplification of a target probe could be carried out when circularized probes become available. These two strict conditions create an ideal detection platform for highly sensitive and specific SNPs detection. By increasing the hybridization temperature and shortening the 3' arm (below the reaction temperature), the discrimination of SNP can be further improved (Pickering *et al*. 2002*,* Faruqi *et al.* 2001). This method for SNPs detection based on DNA ligase-mediated single nucleotide discrimination with consecutive signal amplification by H-RCA has been developed for various groups of pathogenic organisms, including fungi, bacteria, and viruses (Kong *et al*. 2008, Zhou *et al*. 2008, Kaocharoen *et al.* 2008, Wang *et al*. 2009, 2010*).* Recently, the technology has been used to differentiate and to detect two closely related conifer pathogens vectored by the mountain pine beetles (Tsui *et al*. 2010)**.**

Loop mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) is a powerful and novel nucleic acid amplification method that amplifies a few copies of target DNA with high specificity, efficiency, and rapidity under isothermal conditions, using a set of four specially designed primers and a DNA polymerase with strand displacement activity (Notomi *et al.* 2000, Parida *et al.* 2008, Tomita *et al.* 2008). The cycling reactions can result in the accumulation of 10^9 to 10^{10}-fold copies of target in less than an hour. Considering the advantages of rapid amplification, simple operation and easy detection, LAMP has potential applications for clinical diagnosis as well as surveillance of infectious diseases in developing countries without requiring sophisticated equipment or skilled personnel (Mori & Notomi 2009*,* Parida *et al.* 2008).

The technique was first described and initially evaluated for detection of hepatitis B virus DNA by Notami *et al*. (Notomi *et al.* 2000). LAMP assays have been mostly used for the diagnostics of bacteria (Chen *et al.* 2011, Han *et al.* 2011), virus (Wang *et al.* 2011, Zhao *et al.* 2011), and parasites (Ikadai *et al.* 2004, Iseki *et al.* 2007), but it has also been developed for the rapid detection of pathogenic or allergenic fungi. Ohori *et al*. (2006) used the technique for rapid identification of *Ochroconis gallopava*, an emerging fungal pathogen and causative agent of zoonotic infections, while Sun and co-workers, used it for the rapid diagnosis of *Penicillium marneffei* in archived tissue samples (Sun *et al.* 2010a) and of *Fonsecaea* agents of chromoblastomycosis (Sun *et al*. 2010b). Similarly Endo *et al*. (2004) and Tatibana *et al.* (2009) detected the presence of the *gp43* gene in *Paracoccidioides brasiliensis* by LAMP, and Lucas *et al*. (2010) used LAMP for differentiation of *Cryptococcus neoformans* varieties from *C. gattii* based on CAP59 sequences. Recently, Lu *et al.* (2011) utilized the technology for the identification of *Pseudallescheria* and *Scedosporium* species.

Two inner and two outer primers are required for LAMP (Fig. 6A). In the initial steps of the LAMP reaction, all four primers are employed, but in the later cycling steps only the inner primers are used for strand displacement DNA synthesis. The outer primers are referred to as F3 and B3, while the inner primers are forward inner primer (FIB) and backward inner primer (BIP). Both FIP and BIP contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in later stages (Notomi *et al.* 2000). The size and sequence of the primers was chosen so that their melting temperature (Tm) is between 60-65 °C, the optimal temperature for *Bst* polymerase. The F1c and B1c Tm values should be a little higher than those of F2 and B2 to form the looped-out structure. The Tm values of the outer primers F3 and B3 have to be lower than those of F2 and B2 to assure that the inner primers start synthesis earlier than the outer primers. Additionally, the concentrations of the inner primers are higher than the concentrations of the outer primers (Notomi *et al.* 2000, Tomita *et al.* 2008). Furthermore, it is critical for LAMP to form a stem-loop DNA from a dumb-

bell structure. Various sizes of the loop between F2c and F1c and between B2c and B1c were examined, and the best results are obtained when loops of 40 nucleotides (40nt) or longer are used (Notomi *et al.* 2000). The size of target DNA is an important factor that LAMP efficiency depends on, because the rate limiting step for amplification is strand displacement DNA synthesis.

LAMP relies on auto-cycling strand displacement DNA synthesis in the presence of *Bst* DNA polymerase, specific primers and the target DNA template. The mechanism of the LAMP amplification reaction includes three steps: production of starting material, cycling amplification, and recycling (Notomi *et al.* 2000, Tomita *et al.* 2008) (Fig. 6B, C). To produce the starting material, inner primer FIB hybridizes to F2c in the target DNA and initiates complementary strand synthesis. Outer primer F3 hybridizes to F3c in the target and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which forms a looped-out structure at one end (DNA amplification proceeds with BIP in a similar manner). This single stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of a dumb-bell form DNA which is quickly converted to a stem loop DNA. This then serves as the starting material for LAMP cycling, the second stage of the LAMP reaction. During cycling amplification, FIP hybridizes to the loop in the stem-loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem loop DNA with an additional inverted copy of the target sequence in the stem, and a loop formed at the opposite end via the BIP sequence. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA, and one gap repaired stem-loop DNA with a stem elongated to twice as long and a loop at the opposite end. Both of these products then serve as templates for BIP-primed strand displacement in the subsequent cycles, the elongation and recycling step. The final product is a mixture of stem loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Notomi *et al.* 2000, Tomita *et al.* 2008).

LAMP products can be directly observed by the naked eye or using a UV transilluminator in the reaction tube by adding 2.0 µl of 10 fold diluted SYBR Green I stain to the reaction tube separately. Under UV illumination, the gel shows a ladder-like structure from the minimum length of target DNA up to the loading well, which are the various length stem-loop products of the LAMP reaction.

CONCLUSION

Numerous detection methodologies are now available, but regardless of the approach, important questions need to be answered prior to their inclusion into experiments. These include sensitivity, accuracy, robustness, frequency of testing, and cost. Despite many novel technologies being available, challenges remain to identify as yet unculturable fungi, to detect cryptic species, and to characterize the assemblage and diversity of fungal communities in different environments without bias. There is always a need to characterize fungi quickly and accurately. No one knows how many fungal species exist, but sequencing of environmental DNA may improve the accuracy of current estimates (Hawksworth 2001). Next-generation sequencing and pyrosequencing approaches will also provide promising ways of enlarging the scope of molecular-detection studies.

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