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Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

Internal amplification controls have not been employed in fungal PCR hence potential false negative results

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Keywords
entomopathogens, fungi, internal amplification control, medical mycology, mycotoxins, PCR, plant pathology.

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2006/0827: received 8 June 2006, revised and accepted 2 October 2006

doi:10.1111/j.1365-2672.2006.03220.x

Abstract

Polymerase chain reaction (PCR) is subject to false negative results. Samples of fungi with the genes of interest (e.g. a disease or mycotoxin) may be categorized as negative and safe as a consequence. Fungi are eukaryotic organisms that are involved in many fields of human activity such as antibiotic, toxin and food production. Certain taxa are implicated in human, animal and plant diseases. However, fungi are difficult to identify and PCR techniques have been proposed increasingly for this purpose. Internal amplification controls (IACs) will ameliorate the situation and need to become mandatory. These are nucleic acids that posses a sequence which will provide a PCR product (i) using the same primers employed for the target gene, and (ii) that will not coincide on the gel with the product of the target gene. Only one group of workers employed an IAC, to respond to potential inhibition, which was reported in 1995 from this present assessment of numerous reports. Inhibitors in cultures need to be minimized, and secondary metabolites are an obvious source. The fields reviewed herein include medical mycology, mycotoxicology, environmental mycology and plant mycology. The conclusion is that previous reports are compromised because IACs have not been employed in fungal PCR; future research must include this control at an early stage.

Introduction

Fungi are very important organisms. They are involved in antibiotic, food and mycotoxin production: fungal diseases of humans, animals and plants are significant. These eukaryotic organisms may be single cells (yeasts) and/or multicellular (filamentous fungi). They obtain energy by absorption of nutrients through the cell wall and membrane. Many taxa are difficult to identify: They have received a lack of attention from a taxonomic standpoint (Burnett 2003) and remain an enigma (Paterson *et al.* 2004) despite a limited number being studied extensively.

PCR methods for identification of fungi have increased tremendously over the past 15 years, although the use of the technology still lags behind that for bacteria. These procedures have been reviewed for mycotoxigenic fungi (Paterson 2006a) which are unique from this point of view as genes involved directly in the production of the character of interest (i.e. a particular mycotoxin) can be analysed in a few cases. This has the potential for changing concepts of what constitutes a species in terms of a functional taxonomy (Paterson *et al.* 2004, 2006). However, PCR needs to be employed appropriately.

It is a cliché to state that PCR is a scientific breakthrough. Its brilliance should not blind us to potential problems and uncritical use of the methodology (Paterson 2006a). For example, contamination is a drawback. This may from the atmosphere in which the work is being undertaken and seems to be well recognized particularly in medical mycology (Bretagne *et al.* 1995). Unrepresentative sampling is a serious and overlooked problem not only of liquid systems, but also of plants, stored crops, houses, etc. However, this review is concerned with inhibition of PCR.

Negative results can be from (i) the lack of an appropriate gene sequence or (ii) faulty reagents, and/or thermal cycler. A review of this is overdue for fungi following the proposal to make internal amplification controls

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(IACs) mandatory in general microbiology (Hoorfar *et al.* 2004). The need to use one in mycology was realized more than 10 years ago, at least from the inhibition perspective, by one group (Bretagne *et al.* 1995) and they appear to be the only researchers who have used an IAC for the PCR analysis of fungi. As such they require considerable credit. Perhaps the greater awareness was because of the high stakes involved, i.e. directly on people's health. The existence of these authors' work provides evidence (if it was needed) that IACs are necessary and that they are a practical proposition for fungi. The second fundamental message is that fungi need to be grown in such a manner as to minimize potential inhibitors, which may be expected to be secondary metabolites (Paterson 2004). It is surprising that scientists have not anticipated this problem given the wealth of data on secondary metabolite production in culture and the well-known biochemical activities. These issues apply to all organisms but appear not to have been appreciated particularly for fungi. Finally, Ma and Michailides (2006) provide methods for 'eliminating' PCR inhibitors for phytopathogenic fungi, although fail to mention IAC and inhibitors within pure cultures. Furthermore, the authors state that negative PCRs (for mycotoxigenic fungi) indicate that a sample is virtually free of mycotoxins. This is false; samples may contain mycotoxins when there are no fungi, or relevant nucleic acid, present (e.g. Paterson 2006a, 2006b) without even considering false negative results (FNR).

Restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and to some extent, real time PCR etc. have not had the issue of IAC addressed, but there are sufficient reasons to assume that FNR will occur. The use of IAC in these techniques may be more difficult to assess – and rectify. However, the following section represents an assessment of the general situation with respect to fungi.

PCR and internal amplification controls

The reason for IAC is the possibility of FNR in, for example, microbiology. These can arise for various reasons including (i) differences in PCR reagents, (ii) inconsistent and various thermal cycling machines and/or (iii) general inhibition from cultures or substrate (Hoorfar *et al.* 2004). Inhibitors in cultures are likely to be secondary metabolites [see Paterson (2004) for penicillia] which are known to inhibit enzymes. In addition, these compounds have other profound effects or nucleic acid and proteins (e.g. Kamp *et al.* 2005). It is interesting that penicillin had an inhibitory effect on the PCR of *Chlamydia trachomatis* DNA (Lambden *et al.* 2006) as there are no other data on fungal secondary metabolites on PCR assays and provides a model of how this research could be performed. The production of secondary metabolites in culture is qualitatively and/or quantitatively different between taxa and inevitably even between repeated analyses of the same strain (see Frisvad 1998). It would be surprising if researchers did not consider these possibilities at least after they have been made aware of them. Furthermore, a list of the growth conditions used in some reports is provided in Table 1 and, unfortunately, most appear to be reasonable protocols for stimulating secondary metabolite production. Differential extracellular enzyme production by taxa could conceivably have an effect, although it is less obvious what the mechanisms might be.

An IAC is nucleic acid, which can be amplified by the same primers used for the target nucleic acid within the each reaction tube. If the IAC and target products are visualized, then that is a positive result for the gene of interest; in the case of an apparent IAC but no target product then the target gene is not present (however, see below); when the IAC and target products are not apparent on the gel, then the PCR has failed (see Fig. 1). Further purification would be an appropriate response to a failed PCR (Ma and Michailides, 2006), although it may not be successful.

Kainz (2000) found that DNA fragments inhibit polymerases by binding of the enzymes to the nucleic acid and contribute to the plateau effect observed with PCR where the rate of the reaction decreases. Furthermore, Wilson (1997) mentions that nontarget DNA can itself be inhibitory to PCR, although probably only at high concentrations. This is required to be considered for effective IAC. It is logical to suggest that the target DNA could be inhibited selectively so that a product from the target DNA may not be obtained although one could from the nontarget IAC. However, this is hypothetical. Overall, the use of an IAC is a minimum requirement for PCR and particularly those of a diagnostic nature.

An examination of the conditions used to grow the fungi (Table 1) for nucleic acid extraction reveals that only Scherm *et al.* (2005) is in any way designed to reduce secondary metabolism. A medium was used to limit aflatoxin production as an integral part of the experiment (rather than to avoid inhibition of PCR *per se*). Similar information may be valuable for reducing the amount of secondary metabolism for the purpose of PCR. Finally, inhibition of the PCR could occur with secondary metabolite producing bacteria and plants, and the situation with fungi can act as a model for these other organisms. So which studies have employed, or almost universally, not employed an IAC? Some observations are supplied apart from this basic point which also may be of value in a general sense.

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Table 1 Examples of the growth conditions employed in various studies with respect to the possibility of secondary metabolite production and so inhibitors or mutagens

Fungi	Media	Temperature °C	Time (days)	Oxygenation method	Authors
Penicillia	Czapek dox	25	3	Agitation	Pedersen *et al.* (1997)
Fusaria	Sabouraud liquid medium	25	7	Agitation	Jurado *et al.* (2005)
Penicillia	Half strength potato dextrose broth		7–10		Sholberg *et al.* (2005)
Pneumocystis carinii	'100 ml suspension of cell pellet'				Tuncer *et al.* (1998)
Fusaria	'Nicholson *et al.* (1997) method'				Chandler *et al.* (2003)
Tapesia	'Large scale – liquid culture; small scale – scraped from PDA'				Nicholson *et al.* (1997)
Penicillia	Malt extract agar	25	6		Marek *et al.* (2003)
Various: especially aflatoxigenic fungi	Potato dextrose broth		'Freshly growing'	Stationary	Manonmani *et al.* (2005)
Various penicillia	Insufficient information				Colombo *et al.* (2003)
Aspergillus flavus, *Aspergillus parasiticus*	Yeast-extract-sucrose and yeast-extract-peptone	25	5	Stationary	Scherm *et al.* (2005)
Aflatoxigenic fungi + related	Czapek-dox-casein broth	30	3	Stationary	Somashekar *et al.* (2004 a,b)
Aspergillus flavus, *Fusarium poae*	Malt extract broth		3–4	Submersed	Färber *et al.* (1997)
'Clinically significant fungi'	BacT/ALERT FA, BACTEC Plus Aerobic/F, BACTEC Peds Plus/F, BACTEC Lytic/10, BBL MGIT		14 or less		Pryce *et al.* (2006)
Fusaria	Potato dextrose agar		5–7		Jurado *et al.* (2006)
'Environmentally significant'	Sabouraud dextrose agar		At least 10		Dean *et al.* (2005)
Ganoderma	Malt-yeast medium liquid	30	28		Utomo *et al.* (2005)
Ganoderma	Glucose Yeast extract		7–10		Pilotti *et al.* (2000)
Phytophthora	V8 broth	27	3–5	Shaken	Appiah *et al.* (2004)

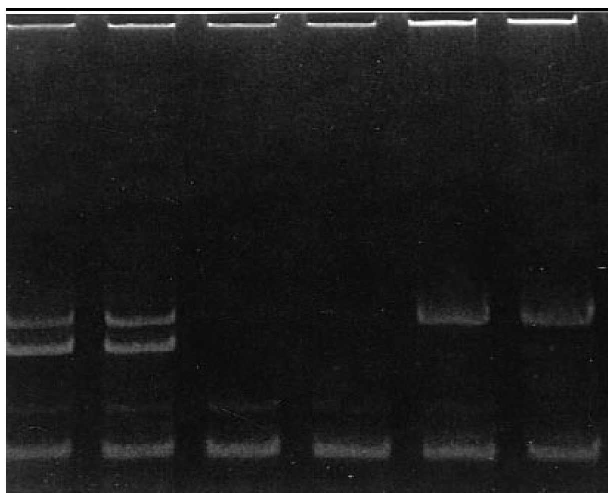

Figure 1 Image of a gel demonstrating from left to right (a) two lanes with a positive reaction with the target and internal amplification (IAC) control being observed, (b) two lanes with a failed reaction where no products were detected, and (c) two lanes with a negative reaction where only the IAC was observed, modified from Bretagne *et al.* (1995).

Studies using an internal amplification control

This is a short section. There is only one group of workers that have employed IAC, although, even here, it was not at all times.

Medical mycology

Bretagne *et al.* (1995) refer to IAC as a 'positive internal control' and fulfil the criteria discussed herein. The authors are to be commended for anticipating this potential problem of inhibition and so early in the development of fungal PCR. However, an IAC (effectively) was not used when testing other fungi in pure culture so they may have been inhibited, rather than the assumption that was made, that they did not contain the relevant DNA. I assume it never occurred to these authors, like so many others (see below), that fungi growing in culture could be affected differentially by inhibition (Paterson 2004) and require equally an IAC. Another point is that very few other fungi were tested in the 1995 report, so interpretations regarding specificity cannot be made conclusively. However, Bretagne *et al.*'s work does tend to support the

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tenants of this present review regarding the requirement for IAC. A good image of a gel is provided with the three possible results demonstrated, i.e. a (i) positive with two bands, (ii) negative with the IAC product but no target and (iii) failed PCR without bands (Fig. 1) (Bretagne *et al.* 1995). It was stated that the predictive value of the PCR is low which is perhaps unsurprising given the previously mentioned shortcomings. Questions of representative sampling, and the difficulty of avoiding contamination of reaction buffers were raised. However, this is not a review of all potential problems: some of these are discussed in Paterson (2006a).

Chambon-Pautas *et al.* (2001) presents an interesting report by employing these above methods to confirm aspergillosis in intestinal tissue (rather than respiratory for example). The figure of the histological section from the patient containing what appear to be hyphae, are an elongated oval-shape with pointed ends which are not reminiscent of aspergilli. In addition, conidiophores were not present raising doubts as to the identification as these structures are required.

Studies not employing an internal amplification control

This is a much bigger section than the previous. The basic problem with these studies is that differences between taxa may not be based on the structure of nucleic acid but on the presence or absence of inhibitors, the effect of which may be transient. This is even without considering the other reasons for FNR (see above).

Medical mycology

IAC were not included in Lass-Flörl *et al.* (2003) and so FNR could have occurred undermining the correlations regarding aspergillosis in mice: as mentioned by the authors, the rate of positive PCR results declined during antifungal treatment, and did not correlate with the underlying invasive disease. For example, it is possible that the antifungal treatment could have inhibited the PCR and hence affect the interpretation of the data. So the lack of correlation could presumably be from FNR where fungal levels were actually not decreasing but rather the PCR was inhibited.

Pryce *et al.* (2003) discuss controls extensively in real time PCR of *Candida albicans* and *Aspergillus fumigatus* diagnosis in humans. For example, they include fungal DNA in preparations of DNA from healthy people for analysis. The issue of IAC *per se* was not addressed. It is conceivable that a positive individual would be considered as negative for the disease which represents the worst possible result. Presumably an IAC could be included in reverse transcriptase-PCR (RT–PCR) which would permit a standard amount of fluorescence and a negative result would give equivalent quantities. A genuinely positive sample would give significantly more fluorescence. For example, an IAC for a real time method is provided in Rodríguez-Lázaro *et al.* (2004) for *Mycobacterium*. Pryce *et al.* (2006) used a positive amplification control of *C. albicans* but not internally and so individual reactions could have been inhibited or the other possibilities could apply as mentioned above. The external control yielded a product that ran to the same position on the gel and so could not be employed as an IAC.

Costa *et al.* (2001) provided details of a RT-PCR method similar to Pryce *et al.* (2003), although in an earlier stage of development. They mention that sensitivities will probably be lower using real samples ('biological samples' compared with water) because of inhibition. It is somewhat surprising that Costa *et al.* do not consider IAC as at least some of the authors were involved with Bretagne *et al.* (1995) – the only group who did employ one. A metabolite other than 'circulatory' DNA could perhaps been measured such as a small secondary metabolite from the fungus. Finally, Tuncer *et al.* (1998) did not use an IAC for *Pneumocystis carinii* in sputum samples and so negative results may have been from, *inter alia*, inhibition.

Food mycology/mycotoxins

Aspergilli

IAC was not included in González-Salgado *et al.* (2005). It is not known whether the negative reactions were accurate and whether the methods described could distinguish consistently between aspergilli of the section *Nigri* on the basis of different DNA as claimed.

Aflatoxin. IAC was not used in the case of Somashekar *et al.* (2004a) so 'differentiation and detection' of *Aspergillus flavus* and *Aspergillus parasiticus* may not be possible consistently. Nevertheless, inhibition is discussed in relation to food components. Furthermore, *aflR* may not distinguish in any case between exclusively sterigmatocystin-producing and not aflatoxigenic species (Paterson 2006a). Somashekar *et al.* (2004b) do not report an IAC. Inhibition in general is not discussed and the negative PCR may have been because of inhibition. In addition, it is possible that fungi which produce sterigmatocystin rather than aflatoxins would yield a positive result as above. There is the somewhat typical confusion between production, compared with detection, of aflatoxins (Paterson *et al.* 2004). When strains are recorded as zero aflatoxin, it needs to be emphasized that this is in fact 'not detected' and not 'not produced'. After all, one

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molecule produced by a culture at some stage in its history is 'is produced' or more accurately, 'was produced'.

Färber *et al.* (1997) did not use an IAC, but did discuss inhibition, particularly from food components and especially from figs. They also demonstrated that fig DNA inhibited the reaction for the target, although at higher concentrations only, with implications for the appropriate use of an IAC. Scherm *et al.* (2005) did not use an IAC for RT-PCR. So the differential expression demonstrated for aflatoxigenic and nonaflatoxigenic strains may be based on FNR. Inhibition did not appear to be considered or discussed in this report. Furthermore, there was ambiguity concerning the production of the so-called 'monoconidial' colonies in addition, e.g. were colonies from a single conidium, or from a single colony forming unit? Again, issues regarding nondetection rather than nonproduction of the aflatoxins needed to be considered.

Manonmani *et al.* (2005) reported a method for the selective detection of aflatoxigenic strains on foods. No IAC was reported although the potential inhibition of the PCR was from the perspective of food components. Again the premise of Manonmani *et al.* cannot be substantiated and other taxa may have had the potential to produce positive results but were inhibited. There were other problems with this report, and other similar ones, regarding co-detection of sterigmatocystin but not aflatoxin fungi as discussed above.

Penicillia

Marek *et al.* (2003) report their assay for *Penicillium expansum* on fruits using the polygalacturonase gene without using an IAC. However, the authors are correct in stating that specific detection is important to ensure microbiological quality and safety of fruits. The problem here, as in other reports, is that the assay may be based on the false assumption that the negatives do not possess the same gene. Of course this might not be problematic in a practical sense – if the non-*P. expansum* strains were negative consistently because of inhibition. However, if the inhibitors were diluted out, then reactions with other species may occur. Inhibition was not discussed in the article. Calderon *et al.* (2002) describe a method for determining spores of *Penicillium roqueforti* (a food-related fungus) from an air sampler using PCR. There is no mention of an IAC although the possibility of inhibition of the PCR is discussed. The consequence is that an underestimation of the concentrations of the fungi in air is possible. Colombo *et al.* (2003) provided a method which claimed to distinguish *Penicillium aurantiogriseum* from foods. There was no IAC and so other taxa may have possessed the appropriate genes and some *P. aurantiogriseum* containing samples may be placed into a category of being negative for the fungus if this system was employed. The article is therefore based on a false premise. However, there are other problems with the article such as the English usage and how few strains were tested. The possibility of inhibition is not mentioned in the article. Sholberg *et al.* (2005) did not use an IAC for *Penicillium* blue rot moulds although a negative control of no DNA in the reaction mixture was included. The use of the method for culture-independent PCR (CIP) (Paterson 2006a), for example, with the specific β-tubulin sequences would mean that negative results could be false in line with the general premise under discussion herein. Again IAC were not employed in Dupont *et al.* (1999), and the RFLP patterns could represent inhibition rather than true differences in DNA between *Penicillium camemberti* and *Penicillium nalgiovense*. The conclusion that IAC are required in RFLP cannot be avoided for all the above reasons, as the method is also enzyme-based, although IAC will be more difficult to employ. Pedersen *et al.* (1997) investigated subgenus *Penicillium* with two sets of primers and again an IAC was not used. An overly bold claim is made (in any case) that all subgenus penicillia can be detected. The authors do in fact mention the possibility of inhibitory substances affecting results. Obviously, without an IAC the claim of being able to identify particular taxa is unreliable. Finally, the images of the gels provided are unclear.

Aspergilli/penicillia

Ochratoxin A. Dao *et al.* (2005) describe a method for detecting ochratoxin A (OTA), and citrinin, producers (see Paterson 2006a). However, an IAC was not employed so little can be said regarding the negative results except that they could be false: some or all of the strains tested may not yield a PCR product because of inhibition of the reaction. In addition, the images of gels are unclear. Sartori *et al.* (2006) report separating OTA-producing species of *Aspergillus* particularly in relation to coffee but without using an IAC. Obviously, from what is being discussed herein, the negative reactions may have been false. The separation of the three species may be based on a false premise that the DNA was different. Negative results from coffee using CIP would also be compromised. It is noticed that *Penicillium* species were not considered, two of which are OTA producers as was determined previously, although others are now implicated (Vega *et al.* 2006; Paterson *et al.* 2004). A consideration of inhibition in general is not provided. Geisen *et al.* (2004) did not use an IAC and so the negative strains tested with the primers may have been inhibited and not specific for *Penicillium nordicum* in terms of the nucleic acid involved. In addition, none of the fungi tested were known citrinin but not OTA producers which may have

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provided false positive results. Part of the OTA molecule is essentially citrinin and so citrinin producing fungi, but not OTA ones, could react (Paterson 2006a). Bogs *et al.* (2006) propose a method for differentiating the OTA-producing species *P. nordicum* and *Penicillium verrucosum*. As there was no IAC, the usual provisos apply concerning negative results and nonappearance of bands. It is noticed that *Aspergillus ochraceus* was completely negative which may be related to inhibition. Finally, Patiño *et al.* (2005) do not mention IAC for separation of *Aspergillus carbonarius* from *A. ochraceus*, strains of which produce OTA. In line with what is being discussed herein, FNR are possible. There was no discussion of inhibition in general in this report.

Patulin. This compound can be considered to be a model mycotoxin and has recently had statutory limits applied to its concentration in fruit products in the European Union. Much of the work on patulin as a mycotoxin using the isoepoxydon dehydrogenase (*idh*) gene and in terms of a diagnostic method has been based on the present author's work. However, an IAC was not employed in the reports (Paterson 2004, 2006a,c). Nevertheless, the possibility of inhibition at least was recognized from the inception of the work and so possession of the gene was correlated with actual detection of patulin. Obviously it was important that there were no cases of negative *idh* and positive patulin detection. An article that largely discusses inhibition from secondary metabolites was produced (Paterson 2004), and the need to use an IAC was mentioned in Paterson (2006a). In terms of using *idh* as a CIP, it is possible to make overall comparisons of the situation in the field as an initial assessment even without IAC as discussed in Paterson (2006c). That is to say, samples from various orchards, for example, could be screened with *idh* primers and attempts made to correlate frequency of detection with concentration of patulin in the final product of apple juice. This approach could be undertaken with some of the other CIP described for other commodities. However, FNR may occur and there is no doubt that an IAC is the preferred option. Work has now discovered DNAs which could act as an IAC for *idh* (R.R.M. Paterson, unpublished).

Fusaria chemotypes

The situation with fusaria is complex as species concepts have not been fully established and the relationship with pathogenicity and mycotoxin production remains unclear in many cases. Quarta *et al.* (2006) describe a mutiplex PCR to detect chemotypes. In addition, a CIP was described to analyse wheat. IACs were not employed; so there is the possibility that negative results were positive in line with the general thesis of this review. That is, each chemotype was not different at the genetic level. Jurado *et al.* (2006) report a multiple copy PCR for toxigenic fusaria which was employed to reduce false negatives. This represents a reminder that PCR does have its own detection limits. However, IAC was not used casting doubt on the results. Chandler *et al.* (2003) attempted to divide various *Fusarium* taxa on the basis of genes responsible for deoxynivalenol (DON) and nivalenol (NIV) production using PCR, in addition to other techniques including analysis of the strains for the mycotoxins: IACs were not used. However, the article is complex with some characters not being tested. There does not appear to be any mention of inhibition. The authors state that all NIV chemotype isolates studied have functional copies of Tri13 and Tri7, and all DON-producing isolates have both genes disrupted or deleted. An obvious conclusion would be that the DON-producing isolates need to be re-tested with an IAC. It is noted that there are no details of the system used for the toxin analysis. Demeke *et al.* (2005) claim to have developed a PCR assay for routine detection and identification of fusaria. However, the interpretation of the result need to be revised as no IAC was employed in line with what is being discussed herein. Similarly, a CIP is discussed in Jurado *et al.* (2006) for fusaria and in relation to mycotoxin production, but its description as a practical method is premature as IAC was not employed.

Plant pathology

True pathogens (parasites)

To continue the theme, the differences in the PCR results for *Verticillium* (Largeteau *et al.* 2006) could have been related to FNR where the PCR product was not observed for some taxa. This would perhaps be satisfactory if the results were consistent, as mentioned before, and therefore it could be claimed to be a valid character. However, it is usually implicit that underlying principals are of differences in nucleic acid sequences *per se*. *Peronospora* species on sweet basil and sage were tested in Belbahri *et al.* (2005). No IAC was used and so the lack of reaction between *Pneumocystis carinii* and (most of the) other species is questionable until an IAC has been employed.

The nonclassical PCR methods (i.e. RFLP, AFLP) are not reviewed extensively herein. The articles reporting them are not as numerous as those for classical PCR. There are reasons to assume that they will be susceptible to FNR as they are also based on enzymatic reactions. Determining a suitable control is much more difficult where multiple bands of relevance are a result. Dick *et al.* (2006) report that ITS digests of two *Phytophthora* species could have been affected by inhibition. Hinojo *et al.*

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(2004) used RFLP PCR to provide patterns in *Gibberella fujikuroi*. Patterns C and D were possible contenders for inhibition as four enzymes gave no bands to provide these patterns, whereas bands were observed for the other patterns. In addition, two enzymes provided no bands at all and were discounted subsequently from the results. It would have been interesting to determine whether an IAC would have provided a product using these enzymes.

Appiah *et al.* (2004) is another case where the possibility of IAC being required for restriction enzyme cuts needs to be considered. The PCR product has not been digested apparently by *Hae* III in the case of *Phytophthora palmivora* and *Pvu* II for *Phytophthora megakarya* and this may be from inhibition. Low enzyme activity is discussed in the article although largely discounted. It may be possible to discover a nucleic acid that gave appropriate cuts for use in each reaction vessel, if not, then at least a control external to the test cuts could be employed. Dupont *et al.* (1999) did not employ an IAC and the RFLP patterns could have been affected by inhibition. The general conclusion is that they are required.

Plant rots

Some PCR-based methods have been developed for white rot of oil palm by *Ganoderma*. However, the main theme of this review applies to these. Utomo *et al.* (2005) is a case in point where an IAC would have indicated whether the negative reactions for the *Ganoderma* strains were genuine or the result of FNR. As no IAC was included, the conclusions about species are invalid, and the use as a diagnostic is based on a false premise at least. Bridge *et al.* (2000) is another case for *Ganoderma*.

Others

Zhou *et al.* (2000) describe a basic protocol that discussed FNR when examining indoor environments. However, IAC are not discussed or utilized with predictable consequences in terms of false negatives (i.e. they may have been positive). Parfitt *et al.* (2005) used PCR without IAC to differentiate species from and within wood. Therefore, PCR primers designed to discriminate *Creolophus cirrhatus* from other species may not be based on differences at the nucleic acid level. *Hericium alpestre*, *Hericium coralloides* and *Hericium erinaceus* are reported to be distinguishable from other wood decay fungi of the Ascomycota and Basidiomycota, but not from each other. No discussion of inhibition is provided, although a control of reaction mixture without DNA was used.

Dean *et al.* (2005) did not employ an IAC for a PCR of four diverse taxa of the indoor environment. These would have been straightforward to identify in any case. It is not know whether the negative strain data are valid. Only a very small number of the same strains of each species was tested and there appear to limited information on strain history; for example, no strain numbers are provided in many cases.

Entz *et al.* (2005) describe a PCR to separate *Entomophthora* which are insect pathogens. *Metarhizium anisopliae* var. *acridum* was claimed to be different from others of the same genus. A CIP was developed but would not be useful optimally without an IAC, and the authors do mention inhibition from insect components. Intraspecific groupings correlated with geographical origin and 'relative' genetic diversity in *Beauveria* (Aquino de Muro *et al.* 2005), although there were no associations with Sunn Pests using PCR-based techniques. However, these may not relate to nucleic acid differences consistent with what is being discussed herein, as IAC was not employed.

Protocol issues

The work of Bretagne *et al.* (1995) could be employed more generally for *A. fumigatus* as they have determined suitable IAC. However, this is not the case for other fungi and so a considerable amount of time may be required to determine suitable DNAs in these cases. A possible problem with the use of an IAC, apart from the additional work, is that the IAC could itself inhibit the positive samples, although obviously the IAC will be used at the minimum concentration possible. The probability of this form of inhibition occurring may be remote at low concentrations. The report involving inhibition of aflatoxin production (Scherm *et al.* 2005) may provide a useful model for reducing secondary metabolism in general for the purposes of PCR in cultures. From a logical perspective, the negative samples may be required to be analysed again but without an IAC. Ultimately, it is the responsibility of the operator to decide what is appropriate in their circumstances.

Conclusions

IAC are necessary for PCR-related analysis because of the possibility of FNR. Inhibitors could logically be secondary metabolites in the case of cultured fungi on artificial growth media. Environmental inhibitors are also plentiful and are straightforward to conceive of in the case of CIP. The more complex RFLP and AFLP techniques theoretically are more difficult to control than classic PCR. Secondary metabolites could affect the structure of the nucleic acid under analysis hence providing 'false differences'. An IAC would not ameliorate this. The conclusion is that IAC is a basic requirement. It is not clear *a priori* whether an IAC will be straightforward to determine in all cases.

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These possibilities can no longer be ignored by those working in the field.

Acknowledgement

RRMP is funded by grant SFRH/BPD/14923/2004 from Fundação para a Ciência e a Tecnologia, Portugal.

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