Molecular characterization of *Aspergillus* species through Amplicon Length Polymorphism (ALP) using universal rice primers

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Goltapeh, E.M., Aggarwal, R., Pakdaman, B.S. and Renu. (2007). Molecular characterization of *Aspergillus* species using Amplicon Length Polymorphism (ALP) and universal rice primers. Journal of Agricultural Technology 3(1): 29-37.

Aspergillus species are among the most cosmopolitant fungi of high importance in medicine, agriculture and biotechnology. Although correct identification of *Asergillus* species needs traditionally morphological studies, molecular strategies are adopted for researchers to precisely facilitate the identification of these fungi. Here, we report the application of a simple and rapid PCR-based technique, amplicon length polymorphism (ALP) by using of universal rice primers.

Introduction

Aspergillus species are among the most ubiquitously found mold fungi throughout the world of high importance in medicine, agriculture and biotechnology. Diseases like allergic bronchopulmonia, mycotic keratitis, otomycosis, nasal sinusitis, and invasive infections are associated with *Aspergillus* species (Beck-Sague and Jarvis, 1993). The most severe disease is the invasive aspergillosis (IA) found mostly with the patients administered with immunosuppressive agents, and of the mortality rate close to 100% (Beck-Sague and Jarvis, 1993; Denning, 1996; Nucci *et al*., 1997; Turenne *et al*., 1999). Early recognition of invasive fungal infection and treatment with appropriate antimycotic drugs are crucial factors in the reduction of invasively disseminated disease (von Eiff *et al*., 1995), however, traditional identification of the causal fungi through morphologic and metabolic characterization takes days to weeks of laborious, time-consuming work and requires significant

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technological expertise (Henry *et al*., 2000; Turenne *et al*., 1999). Some methods like blood culture systems fail to detect most disseminated mycoses like many systemic candidiases (Goodrich *et al*., 1991; Thaler *et al*., 1988) and most cases of invasive aspergilloses (Wald *et al*., 1997). Moreover, the occurrence of *Aspergillus* species less susceptible to chemotherapeutants like amphotricin B than *A. fumigatus* are becoming more common, and make an accurate identification of species crucially important (Hinrikson *et al*., 2005).

Beside causing direct pathogenesis, *Aspergillus* species also produce various types of toxic secondary metabolites, mycotoxins and cause non-contagious mycotoxicoses. Out of all different types of mycotoxins, aflatoxins produced by *A. flavus*, and *A. parasiticus* are the most important, and the consumption of the foods and nutritional materials contaminated with aflatoxins considerably endangers health of human, livestock and birds.

Aspergillus species also produce a range of other mycotoxins like ochratoxins, xanthocillin (hepatotoxic), fumagillin, sterigmatocystin, patulin, aspertoxin, gliotoxin, viriditoxin, fumitremorgins, citrinin, verruculogen, versicolorin, terrain, Austin, emodin, malformin, etc. (for further information refer to *Aspergillus* website available online in http: //www.aspergillus.org.uk/secure/secondary_meta). Discrimination between mycotoxigenic and non-mycotoxigenic species of *Aspergillus* is an important step in the quarantine programs for agricultural products. For instance, while *A. niger* and *A. tubingensis* are morphologically very similar, the former has been reported as ochratoxin A producer. However, the latter is non-mycotoxigenic (Magnani *et al*., 2005). Furthermore, it would be of relevant to distinct between non-mycotoxigenic and mycotoxigenic species as the non-toxigenic species like *A. tubingensis* could be potentially relevant to the biological detoxification of fungal toxins (Magnani *et al*., 2005), as reported before with some non-ochratoxigenic strains of *A. niger* (Varga *et al*., 2000).

Aspergilli have been used for the production of an array of enzymes like proteases for flavor modifications, and organic acids as citric acid, and gluconic acid. Members of *A. flavus* group are used for preparation of oriental beverages like Miso. Control of the unwanted contaminations during large-scaled bio-industrial fermentations necessitates fast and exact identification of the *Aspergillus* species.

Therefore, considerable attempts have been carried out to develop fast and precise molecular techniques instead of laborious and time-consuming mostly based on morphological traits. Most of the molecular methods rely on the determination of the DNA amplicon sequence and its sequence comparison with those available from GenBanks. Here, we want to check the possibility of

molecular specification of aspergilli through amplicon length polymorphism obtained by universal rice primers.

Materials and methods

Fungal material and DNA isolation

Pure cultures of 48 isolates of *Aspergillus* species were obtained from Indian Type Collection. The characteristics of these isolates have been briefly indicated in the table 1. Fungal cultures were grown and maintained on Czapek Dox Agar. Single conidia of each isolate were obtained by serial dilution (1×10^7) from the 3 day CDA cultures. Monoconidial cultures were grown in Czapek Dox broth (NaNO3 2 g/l, KH2PO4 1 g/l, $MgSO_4.7H_2O$ 0.5 g/l, KCl 0.5 g/l, $FeSO_4.7H_2O$ 0.01 g/l, sucrose 30 g/l, distilled water 1000 ml) with a pH adjusted at 6.5 by 1 N NaOH / 1 N HCl, and incubated at 28± 1 °C on continuous shake of 115 rpm for 7 days. Mycelial mass was filtered through a filter paper (Whatman No. 1), washed 3 times with sterile water, air-dried and kept at -20°C till DNA extraction. Genomic DNA was extracted from mycelium following cetyltrimethylammonium bromide (CTAB) method. 2 g of mycelium were transferred to a mortar and ground to a fine powder in liquid nitrogen. 20 ml of extraction buffer (1 M Tris-HCl pH 8.0, 5 M NaCl, 0.5 M EDTA pH 8.0, 5 g CTAB with the final volume adjusted to 250 ml) was added to the finely powdered mycelial mass. The suspension was incubated at 65°C for 1 h. Then, an equal volume of chloroform: isoamyl alcohol (24: 1) was added to the suspension, centrifuged at 10000 rpm for 20 min. Supernatant was precipitated with 0.6 volume of ice-cold isopropanol and 0.1 volume of 3M sodium acetate (pH 5.2), and spinned at 11000 rpm for 15 min. DNA was washed with 70% ethanol, dried and re-suspended in TE buffer (10mM Tris-HCl, pH 8.0, 1 mM EDTA).

Universal rice primers and polymerase chain reaction

The sequences of 6 URP primers, 20 oligo-nucleotides each were originally derived from repeat elements from weedy rice by Hang *et al*., 2002. The primers were synthesized by Genetix (for primer sequences, see Table 2). PCR was performed using a TG radiant thermal cycler (Biorad).

Table 1. Sources of isolates of *Aspergillus* species used for molecular analyses.

Species	Place of collection	Year of isolation	ITCC Number (Isolate Code)
A. sulphureus	Maize rhizosphere, New Delhi (ND)	1970	1412 (5)
A. suphureus	Soil, West Bengal	1999	4881 (6)
A. sydowii	*Dolichos* sp. seed, Nainital	1977	2393 (10)
A. sydowii	Air, ND	1942	308 (11)
A. parasiticus	Karnal	1989	3934 (1)
A. parasiticus	Soil, Madras	1978	2626 (13)
A. ochraceus	Soil, Allahabad	1978	2623 (12)
A. niger	NRRL 599	1943	363 (16)
A. niger	Waste of sugarcane industries, Chandigarh	2001	5579 (18)
A. niger	Soil, Hyderabad	2000	5073 (19)
A. japonicus	Soil, IARI, ND	2000	4939 (25)
A. nidulans	Wheat grain, ND	1975	2010 (2)
A. fumigatus	Soil, Cedrus pine, Hyderabad	2000	5075 (28)
A. fumigatus	Soybean seed, ND	1985	3474 (29)
A. candidus	Wheat grain, ND	1975	2004 (20)
A. candidus	Urid seed, ND	1982	3166 (21)
A. candidus	*Achrus zapota*, Muradabad	1978	2593 (22)
A. candidus	Mouse dung, ND	1980	5428 (23)
A. flavus	Insect, CPCRI, Kerala	1998	4793 (17)
A. flavus	Soil,	1939	298 (18)
A. flavus	Maize grain, ND	1975	2008 (19)
A. oryzae	*Glycine* seed, Nainital	1977	2398 (24)
A. terreus	ND	1942	297 (14)
A. terreus	ND	1986	3550 (15)
A.terreus	Soil, Hyderabad	2000	5074 (16)

Each PCR reaction contained 50-100 mg genomic DNA, 0.2 µM of each primer, 0.2 mm of each dNTP, 0.8 U *Taq* DNA polymerase [MEB (Promega)], 1 × PCR buffer, and 2.5 mM MgCl2 adjusted to the final volume of 25 µl. The profile applied for amplification was as: 1 cycle of 4 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C; 1 cycle of a final extension for 7 min at 72°C. The URP-primed PCR products were separated by the electrophoresis on a 1.5% (W/V) agarose gel in TAE buffer, and visualized by staining with ethidium bromide.

Table 2. Universal Rice Primers used in the molecular studies on *Aspergillus* species, and their sequences.

Primers	Sequences (5' ----- 3')
URP-1F	ATCCAAGGTCCGAGACAACC
URP-2F	GTGTGCGATCAGTTGCTGGG
URP-4R	AGGACTCGATAACAGGCTCC
URP-6R	GGCAAGCTGGTGGGAGGTAC
URP-9F	ATGTGTGCGATCAGTTGCTG
URP-13R	TACATCGCAAGTGACACAGG

Statistical analysis

The DNA fingerprints obtained with the isolates of *Aspergillus* species were scored for the presence (1) or absence (0) of the DNA bands of various molecular weight sizes obtained through PCR-amplification reactions based on different universal rice primers. Data in the form of a binary matrix, and were analyzed to obtain Jaccard's coefficients among the isolates by using the SAHN clustering program. The un-weighted pair-group method with an arithmetic average (UPGMA) algorithm and the software, NTSYS-pc (V. 2.0; Exeter Biological Software, Setauket, NY, USA; Rohlf, 1993) were used for the generation of the dendrogram.

Results and discussion

We used universal rice primers (URPs) to study the possibility of the molecular characterization of different *Aspergillus* species. These primers, - primarily generated from repetitive sequence of Korean weedy rice- were first used by Korean scientists to fingerprint diverse genomes from various organisms including, bacteria, fungi, mammalians, birds and fishes. They concluded that PCR approach using URPs would be useful for studying DNA diversity of most eukaryotic or prokaryotic genomes, especially at inter- and intra-species levels (Kang *et al*., 2002). Jana *et al*. (2005a) took advantage of URPs to study the SSR-based genetic diversity in the charcoal root rot pathogen, *Macrophomina phaseolina*, and perceived the value of such microsatellites in the populational studies as a useful step towards the identification of the potential isolate diagnostic markers, specific to the hosts considered in their studies, namely, soybean, and cotton; and addition of the isolates from another host plant, i.e. chickpea, not only did not reduced the value of URPs, but also

demonstrated their high sensitivity and technical simplicity for uses in genetic variability assays in *M. phaseolina* (Jana *et al*., 2005b).

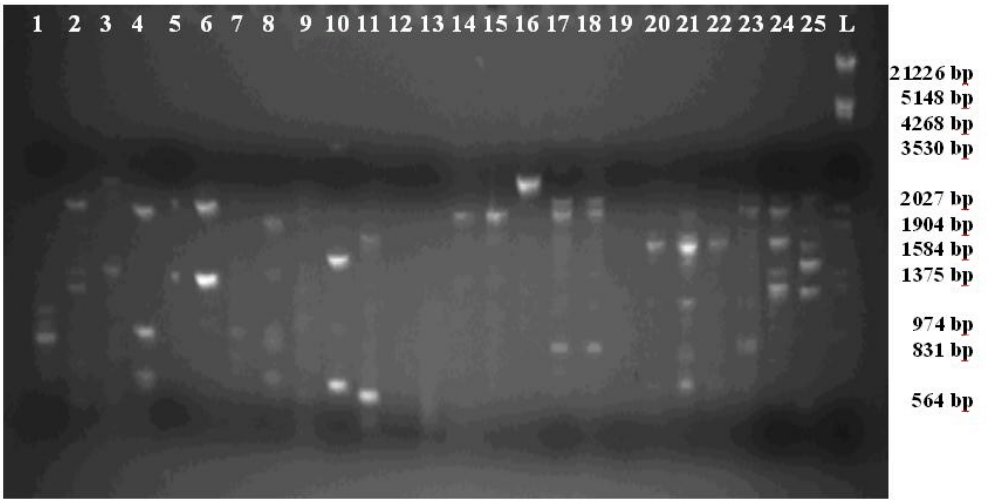

Fig. 1. Banding pattern of different *Aspergillus* isolates resulted from PCR reactions primed by URP-13F primer

So far these primers have been used for sensitive detection of *Pectobacterium carotovorum* ssp. *carotovorum* (Kang *et al*., 2003), and on various agricultural plants (Kang *et al*., 1998). The method has been used in the molecular analysis of genetic variability in *Fusarium* species (Prasad *et al*., 2005). Therefore, we tried the method with *Aspergillus* species to study the possibility of their fast and precise molecular characterization needed in practice. Studies on the banding patterns in different strains and species indicated that all the studied isolates were at least of 70% similarity. *A. oryzeae* had the least similarity (70%) to other *Aspergillus* species, and made a separate branch, however, other species did not locate in distinct branches. Therefore, it seems that the genetic diversity and its development in this genus is so high, so that discrimination of species based on the banding patterns resulted from PCR-amplification with URPs remains so difficult. Instead, the method seems very useful for the studies on the intra-specific diversity in *Aspergillus* species. Three isolates of *A. terreus* occupied three different positions in the dendrogram obtained through data analyses made taking advantage of NTSYS-pc software. The same was true with two different isolates of *A. sydowii*, and *A. fumigatus*. Isolates of *A. parasiticus*, *A. sulphureus*, and *A. ochraceus* were more than 90% similar in their banding patterns. An isolate of *A. sulphureus* was completely similar to *A. ochraceus*, implying the question that if the

former microscopically performed morphological identification was made in a correct manner.

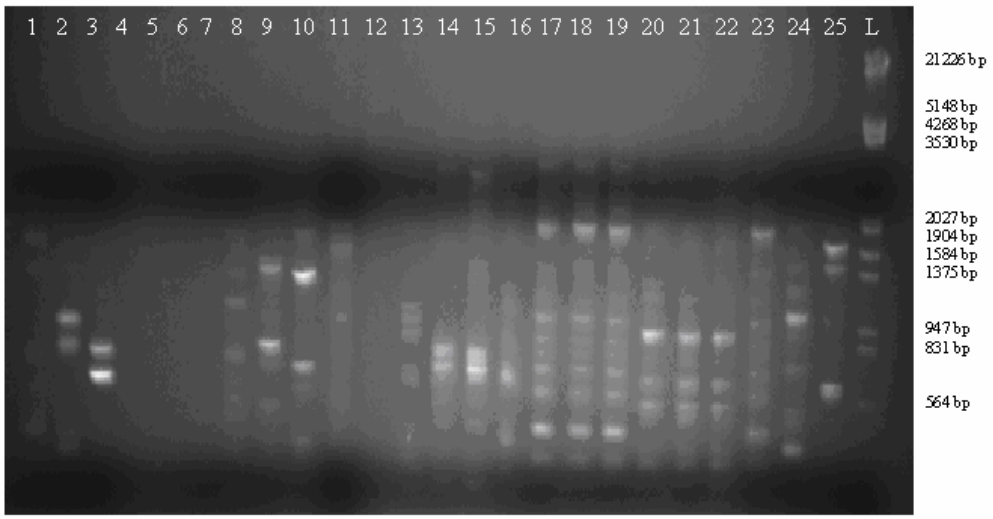

Fig. 2. Banding pattern of different *Aspergillus* isolates resulted from PCR reactions primed by URP-2F primer

Also, it might be possible that similar bands with the same molecular weights are of different in their sequences, therefore, the method can be more improved in its precision through the application of the technique, double gradient gel electrophoresis (DGGE), that makes it possible to discriminate bands of similar molecular weights but various sequences. Our findings are in the agreement with those found by Prasad *et al.* (2005) resulted from their studies on *Fusaium* species. The different isolates, all isolated from safflower plants from different areas of India, and belonged to the single form-species of *Fusarium*, *F. oxysporum* f. sp. *carthami* occupied different positions in three distinct groups with a little inter-group similarity less than 25%, that confirms the idea that the method is not practically useful for the molecular characterization of fungal species with higher genetic diversities, however, still of considerable importance in the molecular discrimination of the different isolates of a fungal species. Such a method can be used with more reproducibility compared with of RAPD, where primers only of 5-10 nucleotides are used to prime PCR reactions.

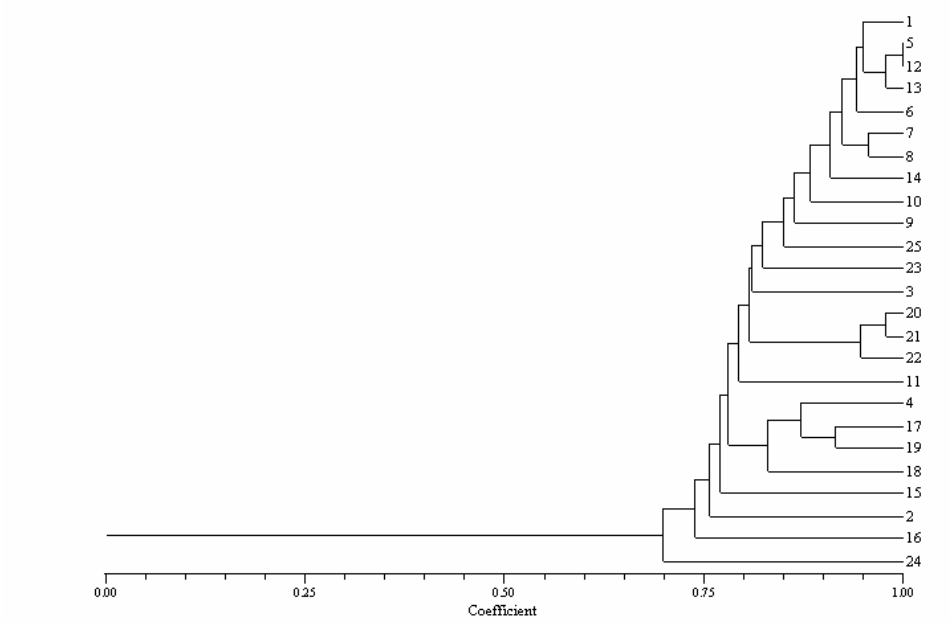

Fig. 3. Combined phenogram of URP markers of 25 isolates of various *Aspergillus* species constructed using NT-SYS. The URPs used were 2F, 2F, 9F, 4R, 6R, and 13 R

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(Received 3 May 2007; accepted 18 May 2007)