

Molecular Divergence and Identification of *Aspergillus* Species in Dry Fishes of Gujarat, India

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Abstract The fungal infestations have become significant cause for the quality and economic loss. They often cause infections to the handlers and consumers. Some of the fungal species are potent allergens, mycotoxin producers and can even cause superficial and pulmonary infection. Reports of previous studies are restricted to genus level due to overlapping of morphological characters. So, the present study was carried out to identify the predominant fungal species prevalent in fishes of west coast region by molecular technique. Totally, 24 sundried fishes were collected from the Veraval region of Gujarat and analyzed for the presence of fungal species. Randomly collected fungal strain was initially indentified based on morphological characters and polymerase chain reaction was carried out for the amplification of 18S rDNA gene. The amplicon was sequenced and analyzed using BLAST. The sequence analysis revealed that *Aspergillus flavus*, *A. nidulans*, *A. oryzae*, *A. sydowii*, *A. rugulosa*, *A. niger*, *A. foetidus*, *Lichtheimia ramosa* and *Curvularia lunata* were present in dried fish. The phylogenetic tree was constructed based on DNA sequence dissimilarity and revealed that two clades were formed among the fungal species with one clade belonging to *A. nidulans*, *A. rugulosa* and *A. sydowii* and the other to *A. flavus* and *A. oryzae*. Therefore, it is concluded that, in west coast region *A. flavus* is a dominant

species observed in dry fish which is potent aflatoxin producers and highly carcinogenic in nature. So, handlers need stringent hygienic practices with suitable processing and packaging technique to control the fungal contamination.

Keywords Dry fish · Fungus · *Aspergillus* · 18S rDNA · Gujarat

Introduction

Dry fish processing is a common practice of Gujarat coastal region and about 20 % of the catch is being processed regularly for domestic and overseas consumption. Totally, 11 export units and 350 domestic dry fish units are involved in processing of dry fish in Gujarat state. About 5000 MT of dry fish per season is regularly exported from India, which accounts for about 80 % of total exports from Gujarat. The consumption of dried fish is about 32 % of the total marine landings in India which is second to fresh fish consumption [1, 2]. About 17 % of the total seafood catch is being used for the production of dry fishes in India [3]. The dried fish product exported from India during 2010–2011 period was 79,059 tonnes which contributed 10 % of total seafood export [4]. Bombay duck (*Harpodon nehereus*), ribbon fish (*Lepturacanthus savala*), shrimp (*Parapenaeus stylifera*), cat fish (*Arius maculatus*), leather jacket (*Aluterus monoceros*), silver bellies (*Gerres subfasciatus*), anchovies (*Stolephorus indicuse*), soles (*Cynoglossus macrolepidotus*, *Paraplagusia bilineata*) and horse mackerel (*Trachurus japonicus*) are some of the common species processed in Gujarat coastal region for dry fish production. The quantity of dry fish export from each unit varies from 30 to 75 MT per month and its price varies

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from Rs 80 to 220 per kg. Based on consumer's preference, dry fishes are being processed with or without salt treatment. The quality of dry fishes is affected by the microorganisms, fungal infestation, insect and rodents, unhygienic handling, poor quality of salt and water used for processing and open sun drying [3, 5, 6]. It badly affects the quality by alteration in flavour, texture and loss of nutrient thus leading to huge economic loss. This can often cause infection to the handlers and consumers [7, 8] and loss of export potentials [9]. Fungal growth on dried fish indicates the onset of spoilage and deterioration of the product [10].

Researchers have reported that the common fungal genera prevalent in dry fishes are *Aspergillus*, *Rhizopus*, *Penicillium*, *Absidia* and *Mucor* [11]. Fungal infestations pose a public health problem of possible contamination with mycotoxins [12]. Several studies have reported the decline in quality of dried fish being affected by fungal growth [13]. *A. flavus* is an opportunistic animal and human pathogen causing aspergillosis (allergic: asthma, alveolitis and allergic bronchopulmonary aspergillosis; saprophytic and invasive colonization: pulmonary and extrapulmonary and liver cancer) and can become fatal in immunocompromised individuals [14, 15]. Prolong intake of low doses of aflatoxins causes chronic aflatoxicosis, resulting in impaired food consumption, stunted growth, immune suppression and liver cancer [16, 17]. During surgical procedures nosocomial infections can occur. Secondary transmission of fungal spores may occur from wounds or by consumption of contaminated plant material [17]. The fungal infections that are caused by are inhalation of fungal spores from contaminated food and feed have been reported worldwide [18]. Economic loss due to fungal contamination are more than a billion dollars in US [19] and the loss in developing countries is considered to be more severe [19, 20]. Consistent intake of these aflatoxins in dried fish products may pose potential public health hazard to the consumers.

Identification of fungi is mostly based on morphological characters and microscopic examinations. These methods are generally restricted up to generic level and hence it is highly difficult to identify them up to the species level through morphological characters besides it usually take 5–7 days for adequate growth; therefore the reliability of the data is also questionable. But, the recent advent of DNA based identification has provided a very clear identification of the fungus up to species level. Identification of fungus up to species level is important because, fungus produces various types of mycotoxins. Production of these mycotoxins varies from species to species i.e., some may produce higher amount of mycotoxins and others producing less. Different types of mycotoxins cause different illness to the humans like

allergy, hepatotoxicity and asthma [21]. Since mycotoxin production varies at the species level, it requires more specific technique to identify the fungus up to species level. Moreover, *Aspergillus* is most commonly present in the humid and tropical countries. Knowledge of extent of prevalence of particular fungal species is very essential to determine the type of toxin production in the region. In this connection very limited published data is available on prevalence of dry fish fungal species in the Gujarat region. Hence, present study is planned to identify the most prevalent species in Gujarat region using conventional and molecular tools.

Material and Methods

Sample Collection

A total of 24 sun dried fish samples like *Harpadon nehereus* (Bombay duck), *Lepturacanthus savala* (ribbon fish), *Parapaeneus stylifera*, (shrimp), *Aluterus monocerus* (leather jacket), *Arius maculatus* (cat fish), and *Ascetes indicus* (non penaid) were collected from fish drying faculties, local markets and landing centres of Veraval and Nawabandar region of Gujarat during a period of March, 2013–April 2015 for enumeration and isolation of fungus. The dried fish samples were brought to the CIFT laboratory in clean airtight polythene bags to prevent contamination. They were further subjected to identification and enumeration of fungus spp.

Enumeration

The enumeration of fungus was carried out by taking Total fungal count (TFC) on potato dextrose agar using standard methods [22]. A tenfold serial dilution was made using 10 g of dried fish sample in 90 ml sterile normal saline and 0.5 ml was spread aseptically on potato dextrose agar plates in three replicates and plates were incubated at 20 °C for 5 days in dark. After post incubation different coloured colonies were counted and results were expressed in Colony forming units/gram (cfu/g). The distinguished fungal colonies were sub cultured on potato dextrose agar (Difco, USA).

Identification

Selected fungus colonies were streaked on Sabouraud Dextrose Agar (SDA) slants (Difco, USA) and grown for 4–5 days at 20 °C for further identification. The fungal isolates were stained with lacto phenol cotton blue stain and observed using direct microscopic method and identified based on standard keys [10].

Polymerase Chain Reaction (PCR)

Fungal DNA was isolated and purified as per the manufacturer's direction (Invitrogen, USA). PCR was performed with 2.0 µl of DNA, 10 µl of 10X buffer, 4 µl of 10 µM of dNTPs mix, 3.0 µl of 50 pM primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3' and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), 5.0 Unit of Taq polymerase, 2.0 µl of 2.5 mM MgCl₂. The PCR mixture was denatured at 94 °C for 10 min, and PCR (Agilent SureCycler 8800, USA) was carried out with the following cycling conditions: 94 °C for 1 min, 60 °C for 2 min and 72 °C for 2 min for 35 cycles. The final extension was carried out at 72 °C for 10 min. Amplicon of 670 bp was electrophoresed in a 1 % agarose gel and visualized under UV.

Sequence

The amplified product (670 bp) were sequenced commercially at SciGenom Labs, Cochin, Kerala. The concentration of the amplicon was checked in a Nanodrop ND 2000. The amplicon was purified using PureLink purification column (Invitrogen, USA). Sequencing of amplicon with forward and reverse primers was carried out in ABI 3730xl cycle sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low quality bases. The sequences were analysed by a basic local alignment search tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST>). The final confirmation of the fungal species was based on more than 99 % identity to the known fungal species and those of fungal generation was <98 %. It was carried out using bioinformatic tool BLAST of NCBI. Based on maximum identity score first few sequences were selected and aligned using multiple sequence alignment software Clustal W, phylogenetic trees were constructed by the DNA maximum-likelihood (ML) method in the PHYLIP program (Phylogeny Inference Package) and neighbour-joining (NJ) method using the DNASTAR Lasergene 13 (Software Development In, Wisconsin University, Madison, USA). Bootstrap analysis with the Clustal W program was

performed by taking 1000 random samples from the multiple alignments.

Results and Discussion

In the present study, amongst the all dry fish samples collected, ribbon fish had the highest fungal count i.e., 1.5×10^1 – 4.5×10^2 cfu/g, whereas the lower counts were observed in cat fish and leather jacket. Based on the direct microscopic observation using lacto phenol cotton blue staining, it was observed that 80 % of the fungi in the study belonged to *Aspergillus*, 10 % to *Mucor* and 10 % to *Pleosporales*. The most prevalent fungus was *Aspergillus* spp. and was found in almost all the dried fish samples. The results of the fungal counts in different dried fish samples with species of the fungus are presented in Table 1. However, the fungal counts did not exceed the permissible limit in these dried fish samples. The isolates were phenotypically identified according to the macroscopic and microscopic morphology of their colonies in Czapek–Dox agar (Difco) are shown in Fig. 1. Similar to the present findings, Chakrabarti and Varma [23] also reported *Aspergillus niger*, *A. fumigatus* and *A. flavus* in the seafood samples in the west coast of Mumbai. Varied reports are also available for the presence of fungus in the marine dried fish samples [9, 24, 25]. Patterson and Ranjitha [8] and Fredrick et al. [26] found higher fungal counts in the commercially dried fishes in India and Wogu and Iyayi [27] observed almost similar findings in Nigeria. Tatiana et al. [28] reported that the TFC in tilapia ranged from $<1 \times 10^2$ – 4.7×10^4 cfu/g in Rio de Janeiro, Brazil, although collected dry fish samples were free from visible fungus colonies on the fishes. The quality of salted and sun dried fishes is adversely affected by the occurrence of fungi [5] which clearly indicates spoilage of dried fish. The microbial load of $>10^6$ cfu/g sample is considered to be an unacceptable limit from the consumer point of view and becomes unfit for human consumption [29]. However, in the present study the fungal counts did not exceed the permissible limit but the presence of aflatoxin producing

Table 1 The average total fungal counts and *Aspergillus* species in the dried fish samples

Dried fish samples	TFC cfu/g	Fungal species present
Ribbon fish (<i>Lepturacanthus savala</i>)	4.5×10^2	<i>A. nidulans</i> , <i>A. oryzae</i> , <i>A. flavus</i> , <i>A. rugulosa</i> <i>A. foetidus</i>
Bombay duck (<i>Harpodon nehereius</i>)	1.5×10^1	<i>A. nidulans</i> , <i>A. oryzae</i> , <i>A. niger</i>
Cat fish (<i>Arius maculatus</i>)	2.0×10^2	<i>A. sydowii</i> , <i>A. oryzae</i> , <i>Curvularia lunata</i>
Leather jacket (<i>Oligoplites saurus</i>)	2.4×10^2	<i>A. oryzae</i> , <i>A. flavus</i>
Kiddi shrimp (<i>Parapenaeus styliifera</i>)	0.8×10^1	<i>A. nidulans</i> , <i>Lichtheimia ramosa</i>

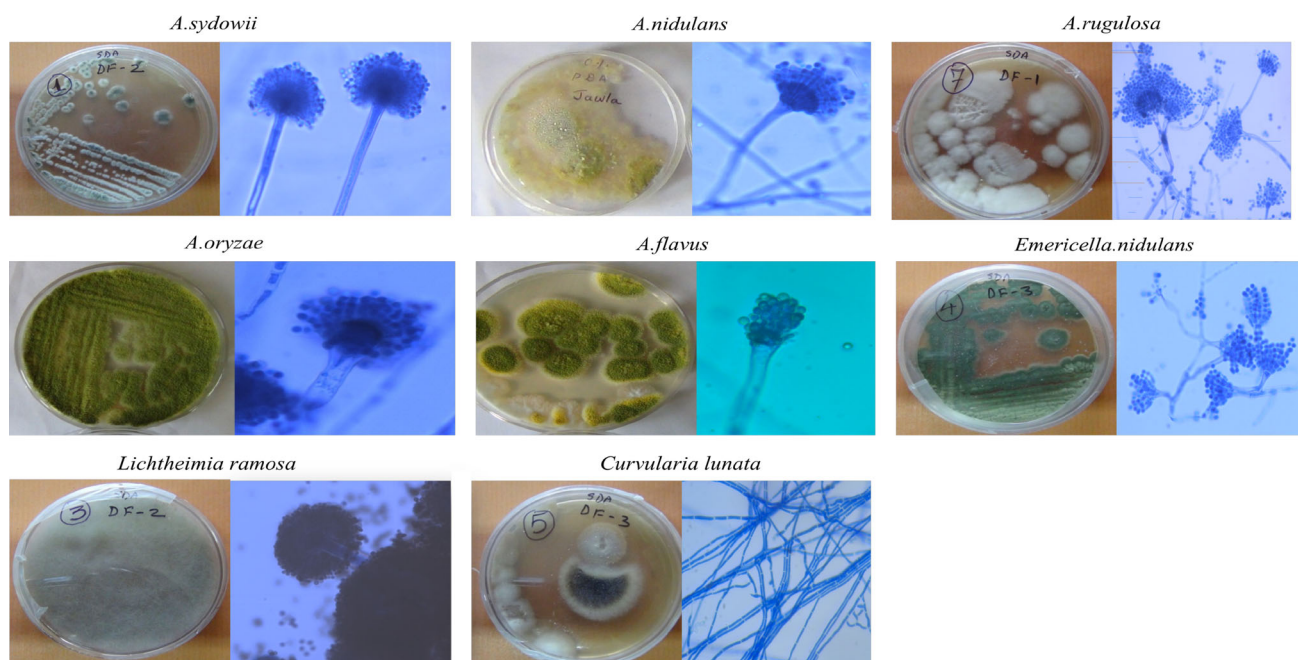
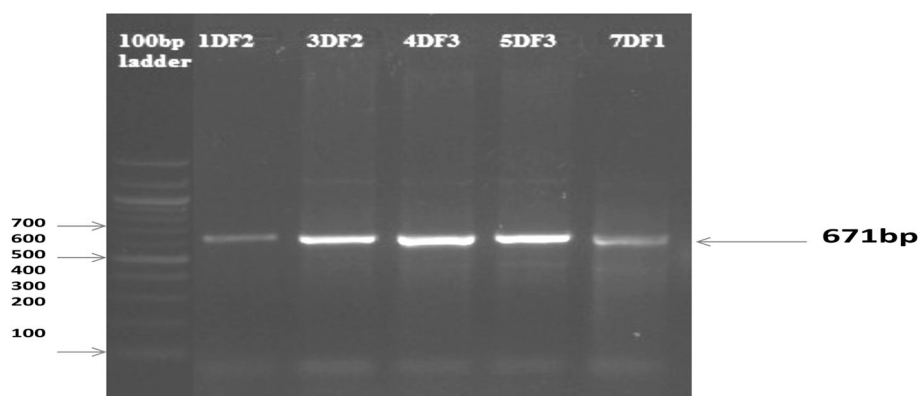


Fig. 1 Fungal species isolated from the dried marine fish

Fig. 2 PCR amplifications of 18S gene with ITS 1 and ITS 4 primers in dried fish samples



Aspergillus species is considered to be a potential health hazards.

Ten to fifteen morphologically and microscopically different fungal isolates were randomly selected and their DNA was subjected to polymerase chain reaction for the amplification of 670 bp in length with the universal primers for the 18S gene with the (internal transcribed spacer) ITS1 and ITS4 regions of the rRNA (Fig. 2). Fungal identification was successful with ITS region with all the samples when aligned with the sequences in GenBank. The identification of fungal *Aspergillus* species supported with universal conserved rDNA sequence methods was suggested by the ongoing *Aspergillus* taxonomy revision are quite accurate and simple as suggested by Balajee et al. [30] and Peterson [31]. The ITS1 and ITS2 region were utilized for diagnostic purpose of fungal species identification in medicine and food ecology [32–35] and also for

phylogenetics and taxonomy purposes. The sequences were blasted in the NCBI following fungal species i.e., *Aspergillus flavus* (3), *A. nidulans* (2), *A. oryzae* (1), *A. sydowii* (1), *A. rugulosa* (1), *Aspergillus niger* (1), *Aspergillus foetidus* (1), *Lichtheimia ramosa* (1) and *Curvularia lunata* (1) were detected in dry fish samples which are shown in Fig. 1. The samples amplified to the fragment size of 650 bp only were taken for the sequence analysis for the accurate identification at fungal species level but *A. niger* and *A. foetidus* could get the sequence of <300 bp. The rDNA sequence differences existing between the *Aspergillus* species are shown in Fig. 3. The least genetic similarity was found between *A. oryzae* with *A. rugulosa* (79.7 %) and *A. flavus* with *A. rugulosa* (79.9 %) as shown in Fig. 2. The highest genetic divergence was found between *A. flavus* with *A. nidulans* (14.0), *A. oryzae* and *A. nidulans* (13.8), *A. rugulosa* with *A. flavus* (13.7) followed

1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	C	C	T	G	Majority
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	G	<i>A. sydowii</i>
1	A	A	C	T	T	C	C	G	T	A	G	G	T	G	A	A	C	C	T	G	<i>A. flavus</i>
1	T	G	A	T	T	C	C	G	T	A	G	G	T	G	A	A	C	C	T	G	<i>A. nidulans</i>
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	C	C	T	G	<i>A. oryzae</i>
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>A. rugulosus</i>
	C	G	G	A	A	G	G	A	T	C	A	T	T	A	C	C	G	A	G	T	Majority
3	C	G	G	A	A	G	G	A	T	C	A	T	T	A	C	T	G	A	G	T	<i>A. sydowii</i>
21	C	G	G	A	A	G	G	A	T	C	A	T	T	A	C	C	G	A	G	T	<i>A. flavus</i>
21	C	G	G	A	A	G	G	A	T	C	A	T	T	A	C	C	G	A	G	T	<i>A. nidulans</i>
7	C	G	G	A	A	G	G	A	T	C	A	T	T	A	C	C	G	A	G	T	<i>A. oryzae</i>
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>A. rugulosus</i>
	G	T	G	G	G	G	T	T	C	C	T	C	-	G	G	G	C	G	C	C	Majority
23	G	C	G	G	G	C	T	G	C	C	T	C	-	G	G	G	C	G	C	C	<i>A. sydowii</i>
41	G	T	A	G	G	G	T	T	C	C	T	A	-	G	C	G	A	G	C	C	<i>A. flavus</i>
41	G	C	G	G	G	C	T	G	C	C	T	C	C	G	G	G	C	G	C	C	<i>A. nidulans</i>
27	G	T	A	G	G	G	T	T	C	C	T	A	-	G	C	G	A	G	C	C	<i>A. oryzae</i>
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>A. rugulosus</i>
	C	A	A	C	C	T	C	C	C	A	C	C	C	G	T	G	A	T	T	A	Majority
43	C	A	A	C	C	T	C	C	C	A	C	C	C	G	T	G	A	A	T	A	<i>A. sydowii</i>
60	C	A	A	C	C	T	C	C	C	A	C	C	C	G	T	G	T	T	T	A	<i>A. flavus</i>
61	C	A	A	C	C	T	C	C	C	A	C	C	C	G	T	G	A	C	T	A	<i>A. nidulans</i>
46	C	A	A	C	C	T	C	C	C	A	C	C	C	G	T	G	T	T	T	A	<i>A. oryzae</i>
1	-	-	-	-	-	-	-	-	-	A	C	C	C	G	T	G	A	C	T	A	<i>A. rugulosus</i>
	C	C	T	A	A	C	A	C	T	-	G	T	T	G	C	T	T	C	G	G	Majority
63	C	C	T	A	A	C	A	C	T	-	G	T	T	G	C	T	T	C	G	G	<i>A. sydowii</i>
80	C	T	G	T	A	C	C	T	T	A	G	T	T	G	C	T	T	C	G	G	<i>A. flavus</i>
81	C	C	T	A	A	C	A	C	T	-	G	T	T	G	C	T	T	C	G	G	<i>A. nidulans</i>
66	C	T	G	T	A	C	C	T	T	A	G	T	T	G	C	T	T	C	G	G	<i>A. oryzae</i>
12	C	C	T	A	A	C	A	C	T	-	G	T	T	G	C	T	T	C	G	G	<i>A. rugulosus</i>
	C	G	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Majority
82	C	G	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>A. sydowii</i>
100	C	G	G	G	C	C	C	G	C	C	A	T	T	C	A	T	G	G	C	C	<i>A. flavus</i>
100	C	G	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>A. nidulans</i>
86	C	G	G	G	C	C	C	G	C	C	A	T	T	C	A	T	G	G	C	C	<i>A. oryzae</i>

Fig. 3 Sequence alignment report of *Aspergillus* species by clustal W

120	A	A	C	T	T	T	C	A	A	C	A	A	T	G	G	A	T	C	T	C	<i>A. rugulosus</i>
	T	T	G	G	T	T	C	C	G	G	C	A	T	C	G	A	T	G	A	A	Majority
193	T	T	G	G	T	T	C	C	G	G	C	A	T	C	G	A	T	G	A	A	<i>A. sydowii</i>
237	T	T	G	G	T	T	C	C	G	G	C	A	T	C	G	A	T	G	A	A	<i>A. flavus</i>
209	T	T	G	G	T	T	C	C	G	G	C	A	T	C	G	A	T	G	A	A	<i>A. nidulans</i>
223	T	T	G	G	T	T	C	C	G	G	C	A	T	C	G	A	T	G	A	A	<i>A. oryzae</i>
140	T	T	G	G	T	T	C	C	G	G	C	A	T	C	G	A	T	G	A	A	<i>A. rugulosus</i>
	G	A	A	C	G	C	A	G	C	G	A	A	C	T	G	C	G	A	T	A	Majority
213	G	A	A	C	G	C	A	G	C	G	A	A	C	T	G	C	G	A	T	A	<i>A. sydowii</i>
257	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	<i>A. flavus</i>
229	G	A	A	C	G	C	A	G	C	G	A	A	C	T	G	C	G	A	T	A	<i>A. nidulans</i>
243	G	A	A	C	G	C	A	G	C	G	A	A	A	T	G	C	G	A	T	A	<i>A. oryzae</i>
160	G	A	A	C	G	C	A	G	C	G	A	A	C	T	G	C	G	A	T	A	<i>A. rugulosus</i>
	A	G	T	A	A	T	G	T	G	A	A	T	T	G	C	A	G	A	A	T	Majority
233	A	G	T	A	A	T	G	T	G	A	A	T	T	G	C	A	G	A	A	T	<i>A. sydowii</i>
277	A	C	T	A	G	T	G	T	G	A	A	T	T	G	C	A	G	A	A	T	<i>A. flavus</i>
249	A	G	T	A	A	T	G	T	G	A	A	T	T	G	C	A	G	A	A	T	<i>A. nidulans</i>
263	A	C	T	A	G	T	G	T	G	A	A	T	T	G	C	A	G	A	A	T	<i>A. oryzae</i>
180	A	G	T	A	A	T	G	T	G	A	A	T	T	G	C	A	G	A	A	T	<i>A. rugulosus</i>
	T	C	A	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	Majority
273	T	C	A	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	<i>A. sydowii</i>
317	T	C	A	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	<i>A. flavus</i>
289	T	C	A	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	<i>A. nidulans</i>
303	T	C	A	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	<i>A. oryzae</i>
220	T	C	A	G	T	G	A	A	T	C	A	T	C	G	A	G	T	C	T	T	<i>A. rugulosus</i>
	T	G	G	C	A	T	T	C	C	G	G	G	G	G	G	C	A	T	G	C	Majority
293	T	G	G	C	A	T	T	C	C	G	G	G	G	G	G	C	A	T	G	C	<i>A. sydowii</i>
337	T	G	G	T	A	T	T	C	C	G	G	G	G	G	G	C	A	T	G	C	<i>A. flavus</i>
309	T	G	G	C	A	T	T	C	C	G	G	G	G	G	G	C	A	T	G	C	<i>A. nidulans</i>
323	T	G	G	T	A	T	T	C	C	G	G	G	G	G	G	C	A	T	G	C	<i>A. oryzae</i>
240	T	G	G	C	A	T	T	C	C	G	G	G	G	G	G	C	A	T	G	C	<i>A. rugulosus</i>
	C	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	T	G	Majority
313	C	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	T	G	<i>A. sydowii</i>
357	C	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	T	G	<i>A. flavus</i>
329	C	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	T	G	<i>A. nidulans</i>
343	C	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	T	G	<i>A. oryzae</i>
260	C	T	G	T	C	C	G	A	G	C	G	T	C	A	T	T	G	C	T	G	<i>A. rugulosus</i>

Fig. 3 continued

	C	C	C	A	T	C	A	A	G	C	C	C	G	G	C	T	T	G	T	G	Majority
333	C	C	C	A	T	C	A	A	G	C	C	C	G	G	C	T	T	G	T	G	<i>A. sydowii</i>
377	C	C	C	A	T	C	A	A	G	C	A	C	G	G	C	T	T	G	T	G	<i>A. flavus</i>
349	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	T	G	T	G	<i>A. nidulans</i>
363	C	C	C	A	T	C	A	A	G	C	A	C	G	G	C	T	T	G	T	G	<i>A. oryzae</i>
280	C	C	C	-	T	C	A	A	G	C	C	C	G	G	C	T	T	G	T	G	<i>A. rugulosus</i>
	T	G	T	T	G	G	G	T	C	G	T	C	G	T	C	C	C	C	C	C	Majority
353	T	G	T	T	G	G	G	T	C	G	T	C	G	T	C	C	C	C	C	C	<i>A. sydowii</i>
397	T	G	T	T	G	G	G	T	C	G	T	C	G	T	C	C	C	C	T	C	<i>A. flavus</i>
368	T	G	T	T	G	G	G	T	C	G	T	C	G	T	C	C	C	C	C	C	<i>A. nidulans</i>
383	T	G	T	T	G	G	G	T	C	G	T	C	G	T	C	C	C	C	T	C	<i>A. oryzae</i>
299	T	G	T	T	G	G	G	T	C	G	T	C	G	T	C	C	C	C	C	C	<i>A. rugulosus</i>
	C	-	-	-	-	G	G	G	G	G	A	C	G	G	G	C	C	C	G	A	Majority
373	C	-	-	-	-	G	G	G	G	G	A	C	G	G	G	C	C	C	G	A	<i>A. sydowii</i>
417	T	C	C	G	G	G	G	G	G	G	A	C	G	G	G	C	C	C	C	A	<i>A. flavus</i>
388	C	-	-	-	-	G	G	G	G	G	A	C	G	G	G	C	C	C	G	A	<i>A. nidulans</i>
403	T	C	C	G	G	G	G	G	G	G	A	C	G	G	G	C	C	C	C	A	<i>A. oryzae</i>
319	C	-	-	-	-	G	G	G	G	G	A	C	G	G	G	C	C	C	G	A	<i>A. rugulosus</i>
	A	A	G	G	C	A	G	C	G	G	C	G	G	C	A	C	C	G	T	G	Majority
389	A	A	G	G	C	A	G	C	G	G	C	G	G	C	A	C	C	G	T	G	<i>A. sydowii</i>
437	A	A	G	G	C	A	G	C	G	G	C	G	G	C	A	C	C	G	C	G	<i>A. flavus</i>
404	A	A	G	G	C	A	G	C	G	G	C	G	G	C	A	C	C	G	T	G	<i>A. nidulans</i>
423	A	A	G	G	C	A	G	C	G	G	C	G	G	C	A	C	C	G	C	G	<i>A. oryzae</i>
335	A	A	G	G	C	A	G	C	G	G	C	G	G	C	A	C	C	G	T	G	<i>A. rugulosus</i>
	T	C	C	G	G	T	C	C	T	C	G	A	G	C	G	T	A	T	G	G	Majority
409	T	C	C	G	G	T	C	C	T	C	G	A	G	C	G	T	A	T	G	G	<i>A. sydowii</i>
457	T	C	C	G	A	T	C	C	T	C	G	A	G	C	G	T	A	T	G	G	<i>A. flavus</i>
424	T	C	C	G	G	T	C	C	T	C	G	A	G	C	G	T	A	T	G	G	<i>A. nidulans</i>
443	T	C	C	G	A	T	C	C	T	C	G	A	G	C	G	T	A	T	G	G	<i>A. oryzae</i>
355	T	C	C	G	G	T	C	C	T	C	G	A	G	C	G	T	A	T	G	G	<i>A. rugulosus</i>
	G	G	C	T	T	T	G	T	C	A	C	C	C	G	C	T	C	G	A	T	Majority
429	G	G	C	T	T	T	G	T	C	A	C	C	C	G	C	T	C	G	A	C	<i>A. sydowii</i>
477	G	G	C	T	T	T	G	T	C	A	C	C	C	G	C	T	C	T	G	-	<i>A. flavus</i>
444	G	G	C	T	T	T	G	T	C	A	C	C	C	G	C	T	C	G	A	T	<i>A. nidulans</i>
463	G	G	C	T	T	T	G	T	C	A	C	C	C	G	C	T	C	T	G	-	<i>A. oryzae</i>
375	G	G	C	T	T	T	G	T	C	A	C	C	C	G	C	T	C	G	A	T	<i>A. rugulosus</i>
	T	A	G	G	G	C	C	G	G	C	C	G	G	G	C	G	C	C	C	G	Majority

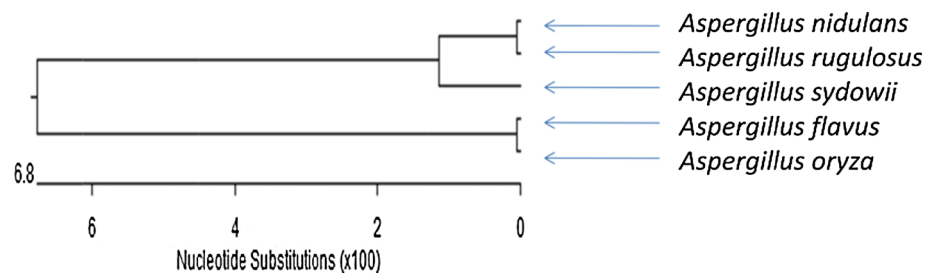
Fig. 3 continued

449	T	A	G	G	G	C	C	G	G	C	C	G	G	G	C	G	C	C	C	G	<i>A. sydowii</i>
496	T	A	G	G	C	C	C	G	G	C	C	G	G	-	C	G	C	T	T	G	<i>A. flavus</i>
464	T	A	G	G	G	C	C	G	G	C	C	G	G	G	C	G	C	C	C	G	<i>A. nidulans</i>
482	T	A	G	G	C	C	C	G	G	C	C	G	G	-	C	G	C	T	T	G	<i>A. oryzae</i>
395	T	A	G	G	G	C	C	G	G	C	C	G	G	G	C	G	C	C	C	G	<i>A. rugulosus</i>
	C	C	G	A	C	G	T	C	T	C	C	A	A	C	C	A	T	T	T	T	Majority
469	C	C	G	A	C	G	T	C	T	C	C	A	A	C	C	A	T	-	T	T	<i>A. sydowii</i>
515	C	C	G	A	A	-	-	C	G	C	A	A	A	T	C	A	A	T	C	T	<i>A. flavus</i>
484	C	C	G	G	C	G	T	C	T	C	C	A	A	C	C	T	T	A	T	T	<i>A. nidulans</i>
501	C	C	G	A	A	-	-	C	G	C	A	A	A	T	C	A	A	T	C	T	<i>A. oryzae</i>
415	C	C	G	G	C	G	T	C	T	C	C	A	A	C	C	T	T	A	T	T	<i>A. rugulosus</i>
	T	T	T	T	T	C	A	G	G	T	T	G	A	C	C	T	C	G	G	A	Majority
488	T	T	C	T	T	C	A	G	G	T	T	G	A	C	C	T	C	G	G	A	<i>A. sydowii</i>
533	T	T	T	T	C	C	A	G	G	T	T	G	A	C	C	T	C	G	G	A	<i>A. flavus</i>
504	T	T	T	C	T	C	A	G	G	T	T	G	A	C	C	T	C	G	G	A	<i>A. nidulans</i>
519	T	T	T	T	C	C	A	G	G	T	T	G	A	C	C	T	C	G	G	A	<i>A. oryzae</i>
435	T	T	T	C	T	C	A	G	G	T	T	G	A	C	C	T	C	G	G	A	<i>A. rugulosus</i>
	T	C	A	G	G	T	A	G	G	G	A	T	A	C	C	C	G	C	T	G	Majority
508	T	C	A	G	G	T	A	G	G	G	A	T	A	C	C	C	G	C	T	G	<i>A. sydowii</i>
553	T	C	A	G	G	T	A	G	G	G	A	T	A	C	C	C	G	C	T	G	<i>A. flavus</i>
524	T	C	A	G	G	T	A	G	G	G	A	T	A	C	C	C	G	C	T	G	<i>A. nidulans</i>
539	T	C	A	G	G	T	A	G	G	G	A	T	A	C	C	C	G	C	T	G	<i>A. oryzae</i>
455	T	C	A	G	G	T	A	G	G	G	A	T	A	C	C	C	G	C	T	G	<i>A. rugulosus</i>
	A	A	C	T	T	A	A	G	C	A	T	A	T	C	A	A	T	A	A	G	Majority
528	A	A	C	T	T	A	A	G	C	A	T	A	T	C	A	A	T	A	A	G	<i>A. sydowii</i>
573	A	A	C	T	T	A	A	G	C	A	T	A	T	C	A	A	T	A	A	G	<i>A. flavus</i>
544	A	A	C	T	T	A	A	G	C	A	T	A	T	C	A	A	T	A	A	G	<i>A. nidulans</i>
559	A	A	C	T	T	A	A	G	C	A	T	A	T	C	A	A	T	A	A	G	<i>A. oryzae</i>
475	C	A	C	T	T	A	A	G	C	A	T	A	T	C	A	A	T	A	A	G	<i>A. rugulosus</i>
	C	G	G	A	G	G	A	A													Majority
548	C	G	G	A	G	G	A														<i>A. sydowii</i>
593	C	G	G	A	G	G	A	A													<i>A. flavus</i>
564	C	G	G	A	G	G	A	A													<i>A. nidulans</i>
579	C	G	G	A	G	G	A														<i>A. oryzae</i>
495	C	G	G	A	G	G	A	A													<i>A. rugulosus</i>

Fig. 3 continued

Table 2 The genetic relatedness among the *Aspergillus* species in dried fish samples

	Percent identity						
	1	2	3	4	5		
Divergence	1	86.8	97.5	86.8	97.6	1	<i>A. sydowii</i>
	2	13.2	86.5	100.0	79.9	2	<i>A. flavus</i>
	3	2.2	14.0	84.2	100.0	3	<i>A. nidulans</i>
	4	13.2	0.0	13.8	79.7	4	<i>A. oryzae</i>
	5	2.2	13.7	0.0	13.7	5	<i>A. rugulosus</i>
	1	2	3	4	5		

Fig. 4 Phylogenetic relationship among the *Aspergillus* fungal species in dried fish samples

by 13.2 % among *A. oryzae* and *A. flavus* with *A. sydowii*. The least genetic divergence was found in *A. sydowii* with *A. flavus* and *A. oryzae* (2.2). There was no sequence divergence among *A. oryzae* with *A. flavus* and *A. rugulosus* with *A. nidulans* (0.0) as shown in Table 2.

The phylogenetic tree was constructed based on DNA sequence dissimilarity and revealed that two clades were formed among the fungal species with one clade belonging to *A. nidulans*, *A. rugulosa* and *A. sydowii* and the other with *A. flavus* and *A. oryzae*. In the first clade, two distinct sub clades were formed wherein *A. nidulans* and *A. rugulosus* in one group and *A. sydowii* in another group (Fig. 4).

In the present study, most of the species belonged to genus *Aspergillus*. This was supported by Rangika and Upali [36] where out of 61 isolates 33 were found to be from *Aspergillus*. Likewise, in Indonesia also higher level of *A. niger* was isolated from the dry fishes [37]. The presence of pathogenic fungi such as *Aspergillus* sp. (*A. flavus*, *A. nidulans* and *A. sydowii*), *Lichtheimia ramosa* and *Curvularia lunata* cause Aspergillosis, mucormycosis and phaeohyphomycosis, respectively. Unhygienic and sanitary practices followed during the traditional method of drying the fishes at various stages are the basis for the fungal contamination, thus posing a serious health hazards to the consumers.

The presence of *Aspergillus*, *Fusarium*, *Rhizopus* and *Mucor* in dried fish along the west coast of India has been reported by various workers [10, 38]. In Cochin market, *A. Flavus* and *A. ochraceus* are dominant [39]; where as in Tuticorin south east coast of India *Aspergillus* and

Penicillium are dominant [40]. Chakrabarti and Varma [23] reported that *Aspergillus niger*, *A. fumigatus* and *A. flavus* are different fungal species found to be dominant in different seasons in the seafood samples. Fungal growth on dried fish indicates the onset of spoilage and deterioration of the product-discolouration, rotting and the production of off odour making food unmarketable incurring huge losses [3, 10, 26, 41]. Improper storage and display of dried fishery products will favour the growth of existing fungal spores and pests may directly inoculate the fungi. Fish are exposed to direct sun light for drying and maintained at ambient temperature for sale but their storage may favour the fungal growth. Dried fishery products from tropical regions have been reported to harbour potentially toxigenic fungi such as *A. flavus* generating potent mycotoxins [37, 42–44]. The optimum temperature for the growth of *Aspergillus* species is >20 °C [10]. The average temperature range of geographical area of the present study was conducted in the range of 20 to 38 °C. Research suggested that post processing contamination can increase the fungal count by more than 106 cfu/g [43]. This is in agreement with the present study.

Conclusion

The present findings reveal the presence of pathogenic fungi in dried marine fish samples. This may be due to the contamination through unhygienic processing while salting, cleaning or drying. So the stringent control measures

are warranted by the use of good quality raw materials such as water and salt, hygienic handling practices, utilization of recommended processing methods, good quality packaging/storing material and cleaned drying place may be considered to improve the quality of the dried fishes. The fungal growth can also be prevented by proper cleaning, salting, drying, reducing the water activity and packaging etc.

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