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ECOLOGY AND TAXONOMY OF PATHOGENIC ASPERGILLI

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1. ABSTRACT

Aspergillus fumigatus and related species are widely distributed in nature. The majority of the species belonging to this genus are saprophytic in nature. Only a few species including *A. fumigatus* are capable of causing diseases in man. These opportunistic agents may cause infection or allergy in susceptible individuals. These fungi also cause diseases in animals, birds, and in plants. In addition, some of the enzymes and metabolic products have tremendous value in industry. A few of the *Aspergillus* species produce potent toxins of the aflatoxin family, which can cause cancer. Toxic death due to aflatoxins has been reported in humans, animals, and birds.

There are currently about 180 recognized species of *Aspergillus*, and these species are placed in 6 subgenera, which are further divided into several sections. The telemorphs belonging to the genera are *Chaetosartorya*, *Dichlanea*, *Eurotium*, *Emericella*, *Fennellia*, *Hemicarpentales*, *Neosartorya*, *Petromyces*, *Sclercleisia*, and *Warcupiella*. This review presents a concise overview of the ecology, taxonomy, and genetics of *Aspergillus* species including their role in plant, animal, and human diseases, production of toxic metabolites, and molecular methods for their identification.

2. HISTORICAL PERSPECTIVE

Aspergilli have always played an important role in life and health of humans. It was Micheli (1), who in 1729 for the first time made a microscopic study of these molds and was able to distinguish stalks (conidiophores) and spore heads. He observed that the spore chains or columns radiated from a central structure to suggest a pattern like that of "Aspergillum" (holy water sprinkler), with which he, as a priest, was familiar. Hence he gave the name *Aspergillus* to these molds. The generic description and some of the illustrations given by Micheli correctly

represent the genus as has been long accepted. The first correct interpretation of structure of conidial head of *Aspergillus* appeared in the work of Corda (2), who described and illustrated his studies from fresh materials. De Bary (3) in 1854 described and illustrated the ascigerous stage (cleistothecia), *Eurotium herbariorum* in *Aspergillus* (*A. glaucus*). In the last 273 years since the work of Micheli, considerable literature concerning *Aspergillus* has accumulated. The early literature was based on microscopic study of specimens collected from natural sources. After De Barry's group began to investigate moulds in comparative culture in the early 1880`s, the study of aspergilli received a great impetus. Later in 1883, Eidam (4) described the ascigerous stage of *A. nidulans*. Wehmer in Germany studied the biochemical aspects of aspergilli (5) and this culminated in the publication of a monograph on the genus *Aspergillus* in 1901 (6). Thom and Church (7) in 1926 attempted to bring together all of the existing literature and published a monograph "The *Aspergilli*", presenting a critical review on the species described. The number of species accepted, either known in culture or determined from existing literature was 60. In the years1929-1930, Tamiya and Morita (8) cited in their "Bibliographe von *Aspergillus"*, 2424 papers which in some way concerned the aspergilli. The role of Aspergilli in industrial processing, decomposition of organic matter in soil, and causing human and animal disease were being increasingly recognized. Thom and Raper (9) did an extensive re-examination of the genus *Aspergillus*, which led to the publication of a monograph *"A Manual of Aspergilli"* in 1945. Eighty species and ten varieties, assembled in 14 groups, were accepted as valid.

Subsequently numerous additional species and varieties were described in the literature, and several ascosporic stages were discovered. Raper and Fennel (10) in 1965 published a comprehensive monograph " The Genus *Aspergillus*", describing exhaustively all the species then known. Numerous additional species and varieties of *Aspergillus* have been described in the literature since 1965. Klich and Pitt published a laboratory guide for identification of common *Aspergillus* species and their teleomorphs (11). A monograph was published by Powell *et al.* (12) on the taxonomy, genetics, and industrial application of the members belonging to the genus *Aspergillus*. Recent treatises on various interrelated subjects of *Aspergillus* have appeared in the literature (13-17). de Hoog *et al*. (18) have presented descriptions of 40 medically important species of *Aspergillus* with illustrations and a key for their identification including molecular features.

3. INTRODUCTION TO *ASPERGILLUS*

The genus *Aspergillus* is distinguished by the presence of a vesicle, a swollen tip of the conidiophore (stalk) bearing phialides (metulae) in single or two series. The phialides bear columnar or divergent (radiate) unbranched chains of conidia. The conidia remain attached to each other by a connective bridge (disjunctor), which may be conspicuous or almost invisible. Conidia are borne in a basipetal fashion, the youngest conidium being at the base and the oldest being at the tip of the chain. Colonies of the fungus are usually rapid growing, powdery, white, greenish yellow, brown or black. Most species of *Aspergillus* reproduce only asexually; sexual state (teleomorph) is known only for a few species but most closely related teleomorphs are known even for species that have no sexual state. The teleomorph of *Aspergillus* is a round, closed structure (called cleistothecium) enclosing numerous asci which contain the ascospores. When the cleistothecium bursts, the asci are spread to the surrounding. The teleomorphs belong to the genera, *Chaetosartorya*, *Dichlanea*, *Emericella*, *Eurotium*, *Fenellia*, *Hemicarpenteles*, *Neosartorya*, *Petromyces*, *Sclercleisia*, *Warcupiella* (Ascomycota, Euascomycetes, Eurotiales, Trichocomaceae). In most cases, asci are 8-spored. Almost all species of *Aspergillus* known to form cleistothecia are homothallic. The cleistothecia vary in size, colour, shape and appearance in different species. The external covering of the cleistothecia in some species e.g. *A. nidulans* is comprised of loose network of hyphae with a large number of terminal or intercalary elliptical or globose, vesiculose cells with very thick walls almost obliterating the cell lumen (Hülle cells).

There are currently about 180 recognized species of *Aspergillus*. Aspergilli are perhaps the commonest group of fungi in human environment and are widely distributed. Many species are found on a wide variety of substrata, including soil, forage products, various types of food products, dust, organic debris and decomposing matter. *Aspergillus* species play an essential role in the recycling of carbon and nitrogen sources. Many species of *Aspergillus* including the important pathogenic species do not have any special nutritional requirements and can grow in simple media, such as glucose-asparagine-phosphate broth, which contains a single protein hydrolysate. *Aspergillus* species can degrade a number of organic components, viz. sugars, fatty acids, proteins, cellulose, pectin, and xylene (19).

4. IMPORTANCE OF ASPERGILLI IN INDUSTRY, AGRICULTURE, AND HUMAN HEALTH

4.1. Industrial importance of aspergilli

Several species of *Aspergillus* have industrial importance. For instance, *A. oryzae* and *A. niger* are widely employed for production of enzymes, including cellulases, amylases, proteases, lipases, and pectinases (19, 20). These fungi are well-suited for large-scale industrial production because of their efficient secretion system. The most widely used enzymes are lipases and proteases, as additives to detergents. Other enzyme preparations from *Aspergillus* are used for malting in the production of brewed and distilled alcoholic beverages, as food additives to aid digestion in livestock and humans, and for degradation of fats and oils for foods and fragrances (20). *Aspergilus niger* is also employed in the production of glucuronic acid by fermentation of corn steep liquor supplemented with glucose, and citric acid by fermentation of molasses. *Aspergillus oryzae*, *A. sojae* and *A. tamari* have been used for over 4000 years in a number of food

processes. For example, *A. oryzae* is employed in the fermentation of soya bean to produce soy sauce (shoyu), miso, and in the fermentation of rice to produce the wine, `sake` (19, 20). Many mold-ripened sausages in Germany and Eastern Europe utilize *Aspergillus* species and are coated with a layer of white mycelium of *Aspergillus*.

4.2. Role of aspergilli in plant infection and production of toxic metabolites

Aspergillus species are considered as weak plant pathogens. *Aspergillus flavus* and *A. parasiticus* have a particular affinity for nuts and oil seeds. The three most important crops affected are peanut, maize and cottonseed. These fungi generally infect plant hosts wounded by insects or other agents (20). Penetration of healthy tissue of plants causing systemic infection has been demonstrated under conditions of stress e.g. drought (20-22). Pre-harvest infection of fruits by *Aspergillus* spp. is a serious problem (21). Systemic infection of developing pea-nut plant by *A. flavus* has been reported (21, 22). Pre-harvest infection in maize is partly dependent on insect damage to cobs, but the fungus can also invade the developing ears (23). The greatest concern of plant infection by *Aspergillus* involves the production of aflatoxin (a polyketide) by *A. flavus* and *A. parasiticus* in stored grains and nuts, a process that is probably initiated frequently by pre-harvest penetration by the fungus (22, 23). Aflatoxins were first discovered after the deaths of over 100,000 turkeys fed with contaminated groundnut meal (24).

Aflatoxicosis is a well-recognized disease in birds, other animals, and humans. Spores of *A. flavus* and other species of *Aspergillus* are often found in outside air. Under stress conditions of drought and high temperature, maize and groundnut may be infected with *A. flavus* leading to contamination with aflatoxin before harvest (22, 23). Both *A. flavus* and *A. parasiticus* produce aflatoxin B_1 and B_2 while *A. parasiticus* produces, in addition aflatoxins G_1 and G_2. The symbols `B` and `G` refer to the blue and green colours produced by these toxins under UV light on thin layer chromatography plates, while the subscript numbers 1 and 2 indicate major and minor compounds respectively (23). Aflatoxin B_1 is widespread in contaminated foodstuffs and is considered a great hazard to human health. It is a type 1 human carcinogen (25). In 1974, an outbreak of hepatitis resulted in 100 deaths from consumption of heavily contaminated maize (26). Both *A. flavus* and *A. parasiticus* grow at temperatures 10 to 43^0C with an optimum near 32-33^0C, aflatoxin being produced between 12 and 40^0C. Reduction of available oxygen by modified atmosphere packaging of foods in barrier film or with oxygen scavengers can inhibit aflatoxin production by *A. flavus* and *A. parasiticus* (25). Another mycotoxin, ochratoxin A (OA), a pentaketide is produced by *A. ochraceous*, *A. carbonarius* and occasionally by other species including *A. niger* (22). *Aspergillus ochraceous* has a natural habitat in dried or decaying vegetation, seeds, nuts and fruits (22). It grows in the temperature range of 8-37^0C, with ochratoxin produced nearly over the entire range. *Aspergillus carbonarius* is also wide spread in tropical foods and can survive sun drying. It grows in the range 10-40^0C (25). OA is associated with both acute kidney disease and cancers of the renal system and urinary tract (27). It has been linked to renal disease in Tunisia and Egypt (25).

4.3. Role of aspergilli in animal infections

Aspergilli have been known to infect the lungs and air-sacs of birds of all types (28, 29). The two major clinical manifestations of aspergillosis in birds are pneumonia in young chicks and chronic pulmono-visceral disease in older birds (28). Outbreaks of acute forms (called brooder's pneumonia) have caused devastating losses of birds in hatcheries (28, 30). In these epizootics, the source of the infecting fungus has been traced to contaminated litter (28). The disease is most commonly caused by *A. fumigatus*, although *A. flavus*, A. *niger*, *A. nidulans*, and *A. terreus* may be occasional causal agents (28, 31). Acute aspergillosis presenting as placentitis has been frequently reported in cattle, and sometimes in sheep, horses and pigs (28). Cases of disseminated invasive aspergillosis caused by A. *terreus*, and *A. deflectus* have been reported in dogs (32-34). Sinus infections due to *Aspergillus* also have been described in dogs (35). *Aspergillus nidulans* has been known to cause mycosis of the guttural pouch (air-filled diverticulum of the Eustachian tube) in horses (36).

4.4. Species of *Aspergillus* pathogenic to humans

A diversity of species of *Aspergillus* have been implicated in human mycoses. de Hoog *et al*. (18) have described 40 species reported as causal agents of opportunistic infections in humans. Many species are thermotolerant and their dry hydrophobic conidia are easily inhaled. Among these, *A. fumigatus* is the most frequent etiological agent followed by *A. flavus* and *A. niger*. Among the remaining species, less commonly or rarely recovered as opportunistic pathogens are *A. clavatus*, *A. glaucus*, *A. nidulans*, *A. oryzae*, *A. terreus*, *A. ustus*, *A. versicolor*, *A. ochraceus* and several others (13, 18, 37). Aspergilli cause a variety of opportunistic infections, such as mycotic keratitis, otomycosis, nasal sinusitis, allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, invasive aspergillosis, which may present in a wide spectrum, varying from local involvement to dissemination. Immunosuppression is the major factor predisposing to development of invasive aspergillosis (37). The frequency of invasive *Aspergillus* infections has increased in recent years due to increasing number of patients receiving aggressive chemotherapy and immunosuppressive agents.

4.5. Aerobiology of pathogenic aspergilli

Aspergillus species are ubiquitous in the environment, especially near decomposing vegetation. It has been suggested that the environment plays a crucial role in the epidemiology of invasive aspergillosis. *Aspergillus* conidia released in the air from several saprophytic sources of the fungus are dispersed in air currents and remain airborne for prolonged periods. *Aspergillus* spores are thus ubiquitously found in air and contaminate anything in contact with air (37). Viable propagules of *Aspergillus* spp (usually conidia) are commonly recovered from both indoors and outside. Inhalation of airborne conidia, either directly or through

intermediate nasopharyngeal colonization is considered a direct cause of pulmonary infection in the immunocompromised host (38). Several studies have been conducted to correlate the airborne concentration of *Aspergillus* and risk of invasive disease and colonization. Increased incidence of aspergillosis and/or increased concentration of airborne *Aspergillus* have been attributed to hospital construction and renovation, excavation, contaminated air filtering systems, fire proofing material, damp wood, potting soil, shower wall, carpeting and season of the year (38-43). In one study (41), the cluster of cases of invasive aspergillosis in a bone marrow transplant unit (BMTU) could not be linked to construction, though construction did increase the rates of *Aspergillus* colonization in the BMTU and the adjacent wards. Outside air conidial levels have been reported to range generally from 1 to 5 cfu m-3 (41). Hospenthal *et al.* (44) recovered *A. fumigatus* and *A. flavus* at an average of 1.83 cfu m-3 air sampled over a period of 54-week period in the oncology wards of hospital. Other *Aspergillus* species were recovered at a mean of 2.38 cfu m-3. There was no correlation of air borne concentration of aspergilli with ward or season nor was there any association linking the six cases of invasive aspergillosis seen during the air sampling period with the changes in recovery of airborne *Aspergillus* (44). On the other hand, Paugam *et al.* (45) were able to demonstrate by molecular typing the identity of *A. fumigatus* isolates obtained by air sampling and the one from a deep-tissue of an invasive case of aspergillosis in an immunosuppressed patient. The importance of optimal physical barriers and air filtration to decrease airborne fungal spores in high-risk units has been emphasized. Oren *et al.* (46) reported that keeping neutropenic patients in a special ward with high efficiency particulate air filtration (HEPA) system eliminated the risk of invasive aspergillosis completely. In view of these reports, it is desirable to have efficient air filtering devices e. g. HEPA filters to aim at reducing *Aspergillus* conidia to zero level in the air of wards housing severely immunocompromised patients.

A few aeromycological studies have also been carried out in relation to suspected cases of hypersensitivity pneumonitis in some occupational groups. Sandhu *et al.* (47) carried out an aeromycological survey in 2 cane sugar mills where a case of hypersensitivity pneumonitis in an employee of one of the mills had originated. *A. fumigatus* accounted for 42.5% of the total aerial fungal colony counts recorded in the mills as against 2% in other localities. Further, the aerial concentration of *A. fumigatus* in the bagasse-containing sites was higher (50.3%) than in the bagasse-free sites (13.5%) in the mills (47).

4.6. Public health importance and virulence factors of *Aspergillus fumigatus*

Aspergillus fumigatus is the most important pathogenic species among aspergilli. It is thermotolerant, can grow over a temperature range from 20 to 520C, optimally at 370C, well at 30-45 0C, and it can survive for long periods of time at 55 0C (37, 48, 49). *Aspergillus fumigatus* is thus abundant in self-heating environments such as hay, corn, and compost. It is also very frequently isolated from herbivore dung, accumulations of bird droppings, bird nests, inadequately heat dried plant material, such as spices or marijuana-cultivated soils and graminaceous seeds, such as those of wheat, barley, oats, rice, sorghum and corn (20, 22, 37). Humans and animals constantly inhale numerous conidia of *A. fumigatus.* The conidia are normally eliminated in the immunocompetent host by innate immune mechanisms. Allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, mycotic keratitis, otomycosis and nasal sinusitis are the only infections observed in such hosts. In the immunosuppressed patients, in addition to these infections, it causes invasive and disseminated aspergillosis (48). *Aspergillus fumigatus* is an important and life threatening aerial pathogen frequently causing nosocomially acquired invasive aspergillosis, typically in the treatment setting of hematological patients. The invasive infections occur most frequently in patients who are neutropenic or have dysfunctional neutrophils (37, 48). Conidia of *A. fumigatus* are airborne and are present in all environments at concentrations between 0.1 and 3 conidia m-3 of air; their small size allows them to reach all levels of the respiratory tract (49).

It has been hypothesized that when A. *fumigatus* undergoes transition from a free-living organism existing on dead organic matter in the environment, to an opportunistic pathogen, it undergoes metabolic reprogramming that induces the expression of specific sets of genes necessary for the growth in the host. Several virulent factors have been identified in *A. fumigatus* (48, 49), that could mediate host tissue adherence, colonization and invasion, and facilitate the escape of the host immune responses. The ability of *A. fumigatus* to bind to extracellular matrix (ECM) of host tissue is considered crucial in the establishment of the fungus and in initiation of infection (50). Several components of ECM such as fibrinogen, laminin, fibrinectin and type I collagen have been proposed as cellular ligands for conidia (50). The conidial hydrophobin helps the fungus to adhere to epithelium. Recognition of some glycoproteins present at the surface of the host tissue (called ligands) also appears to be a crucial step in the initiation of infection (50). Adhesintopes for *A. fumigatus* are located on the outer D domains of the fibrinogen molecule, and in laminin P1 (51). A variety of enzymes produced by *A. fumigatus*, viz. catalase, aspartic protease, metalloprotease, elastase and collagenase help in lung colonization and invasion of host tissue (49). The enzymes phopholipase and ribonucleases destroy host cells, and superoxide dismutase protects the fungus against antioxidants. *Aspergillus fumigatus* also produces two major hydroxymates, N, N`, N" triacetylfusarinine C and ferricrocin siderophores, which can compete successfully with human iron proteins to procure iron to support fungal growth (50).

A monograph by Brakehage *et al.* (52) gives a comprehensive overview of biology, clinical aspects, epidemiology, and molecular biology of *A. fumigatus* infections.

5. CLASSIFICATION AND IDENTIFICATION OF *ASPERGILLUS* SPECIES

The genus *Aseprgillus* is now assigned to the family Trichocomaceae of order Eurotiales in the class Plectinomycetes of phylum Ascomycota (18). Currently species in the genus *Aspergillus* are taxonomically placed

Table 1. Morphological criteria used for identification of *Aspergillus* species

Colony characteristics	Rate of growth
	Pigmentation of the aerial mycelium
	Colour of reverse
	Appearance of the colony margin
	Texture of surface growth
Conidial head	Colour
	Shape, overall dimension
	Distribution of conidial chains in massed upright columns, , or separate radiating conidial chains, or
	a formation intermediate between the two arrangements
Conidiophore	Length
	Diameter
	Wall characteristics
Vesicle	Shape
	Dimension
	Colour
	Fertile area
Phialides	Uniseriate (single row) or biseriate (two rows)
Conidia	Dimension
	Wall characteristics
	Colour
Hülle cells (present or absent)	Shape and colour
Sclerotium or sclerotia like bodies (present or absent)	Form
	Dimension and colour
Sexual stage (Cleistothecia)	Colour, form, dimension and structure
	Ascospores pattern, colour and dimension
Aleuriospores	Present or absent

Adapted from Summerbell (13) and Richardson (37).

in well-characterized subgenera, viz. *Aspergillus*, *Fumigatus*, *Ornati*, *Clavati*, *Nidulantes* and *Circumdati.* Each of these subgenera is divided into several sections containing one or more species (11). For instance, *A. fumigatus* belongs to the section *Fumigati* of subgenus *Fumigati*, while *A. clavatus*, *A. nidulans*, *A. versicolor*, *A ustus* and *A. terreus* are placed in the sections *Clavati, Nidulantes*, *Versicolores*, *Usti* and *Terrei* respectively in the subgenus *Nidulantes*. *Aspergillus glaucus* and *A. restrictus* belong to the sections *Aspergillus* and *Restrictus* respectively, in the subgenus *Aspergillus*. Likewise, *A. flavus*, *A. niger*, *A. ochraceous*, and *A. candidus* belong to the respective sections *Flavi*, *Nigri*, *Circumdati*, and *Candidi* in the subgenus *Circumdati* (11).

A very large number of species in the genus and variations in the morphological characteristics of individual species has created the need for alternative approaches. Determination of isoenzyme patterns has facilitated classification of some species, which are difficult to identify by morphological criteria (53). Other approaches used to delineate species include the presence of extracellular polysachharides (54), biotyping by the yeast killer system (55), the ubiquinone system, especially Q-9 and Q-10 (56), serological typing (57), and pectinolytic enzymes (58). Application of combination of different types of biochemical, physiological and morphological characteristics and molecular approaches to taxonomy of *Aspergillus* has been discussed by several workers (13, 14, 59). This approach has not yet found application for routine species identification of *Aspergillus*. Practical identification is still essentially based on a detailed study of morphological and cultural characteristics of the isolates (10, 11-13, 15-18).

Since only a few species of *Aspergillus* form the sexual state, it is the anamorphic state, which is usually encountered in culture from clinical specimens. Thus identification of most of the species is based on the basis of characteristics of the colonies and a detailed study of the microscopic morphology, especially that of the asexual spore bearing head. The key for identification of 13 species of *Aspergillus* presented here is based on these characteristics. Several monographs and text books are available as aids in the identification of common species and medically important species (11-13, 15-18). The important morphological criteria used in species identification of aspergilli are listed in the Table 1.

5.1. Morphology of *Aspergillus*

Colony characters studied include pigmentation of the aerial mycelium or the underlying medium, rate of growth, appearance of the colony margin, whether heavy or sharply limited or thin and diffuse, smooth and entire or irregularly lobed or submerged and the texture of surface growth, whether velvety (consisting of submerged mycelium, from which only fruiting structures arise above the surface) or floccose (producing a conspicuous aerial felt of branching and interlacing hyphae and conidiophores). The characters of the conidial head include its colour and shape i.e. conidia produced in dry chains forming columns (columnar), or diverging (radiate) observed in a mature sporing culture, colour, size, shape (globose, subglobose or oval) and wall texture of the conidia i.e. smooth-walled or ornamented, ii. shape of the vesicle (subspherical, hemispherical, clavate, or flask-shaped); iii. roughness or smoothness of the conidiophore wall; iv. phialides being in single row (uniseriate) or two rows (biseriate). These morphological features of conidial heads are characteristics of the species and are better studied in slide culture. Examination of several heads in colonies (often on several media) is essential for complete description and interpretation of their size and shape. Cells with extremely thick walls (Hülle cells) of unknown function may be present in certain species of *Aspergillus*. The close and consistent association of globose Hülle cells with cleistothecia of *A. nidulans* suggests that presence of such structures in other species may indicate a potential for cleistothecia formation. True sclerotia consisting of thick-walled, parenchyma-like cells occur regularly in some isolates of *A. candidus*, *A. niger*, *A. flavus* and *A. ochraceus*. Some isolates of *A. versicolor*, *A. wentii* and *A.*

Table 2. A key to the 13 pathogenic species commonly involved in human infections is given below followed by brief descriptions of the species

1a. Colonies some shade of green	2
1b. Colonies not green	7
2a. Vesicles elongate, clavate or club shaped	*Aspergillus clavatus*
2b. Vesicles globose, or hemispherical	3
3a. Colonies uniformly yellow green, conidiophores rough-walled	*Aspergillus flavus*
3b. Colonies yellow green but turning brown with age, conidiophores rough-walled	*Aspergillus oryzae*
3c. Colonies dark green, very restricted in growth, conidial heads long and twisted	*Aspergillus restrictus*
3d. Colonies dark green, sometimes with yellow areas, conidiophores smooth-walled	4
4a. Phialides in one series	5
4b. Phialides in two series	6
5a. Phialides on upper two-thirds of small dome shaped vesicle	*Aspergillus fumigatus*
5b. Phialides on the entire surface of the large globose or club shaped vesicle	*Aspergillus glaucus*
6a. Conidiophores pale brown, conidial heads short columnar, ascospores with two equatorial crests	*Aspergillus nidulans*
6b. Conidiophores hyaline, conidial heads radiate, colonies colour variable from green to yellow green, buff in the same culture	*Aspergillus versicolor*
7a. Colonies white, conidial heads radiate	*Aspergillus candidus*
7b. Colonies black or in shades of gray, brown or ochre	8
8a. Colonies black, conidial heads radiate, globose vesicles entirely covered by phialides	*Aspergillus niger*
8b. Colonies dull gray to charcoal coloured, conidial heads radiate to loosely columnar	*Aspergillus ustus*
8c. Colonies cinnamon brown to sand coloured, conidial heads densely columnar	*Aspergillus terreus*

terreus form pseudosclerotia–sclerotium-like masses of compacted mycelium or discrete but loosely aggregated mycelium.

5.2. Description of the species

The salient gross and microscopic features of the species included in the key are described below as observed on Czapek agar. Standard description of species known primarily by their teleomorph stage, viz. *Neosartorya fischeri*, *N. pseudofischeri*, and *N. spinosa* are given in the reference work of de Hoog *et al.* (18). A key to the 13 pathogenic species of Aspergilli is given in Table 2.

5.2.1. *Aspergillus candidus*

Colonies usually slow growing, 1.5 to 3.5 cm in diam. after 2 weeks at room temperature (24-28 ^{0}C), flat to domed, granular to floccose, persistently white or pale cream to yellow with reverse generally colorless. Conidial heads white to cream, radiate, often appearing loosely columnar. Conidiophores 200-500 µm in length, hyaline, smooth-walled to finely roughened. Vesicles subspherical to spherical, 10-40 µm diam. Phialides biseriate, occasionally uniseriate in smaller heads, covering the entire surface of the vesicle. Sometimes smaller heads may lack the vesicle and resemble *Penicillium* conidiophores. Conidia hyaline, smooth-walled, spherical to subspherical, 2-5-4.0 µm diam. Sometimes reddish purple to black sclerotia may be present.

5.2.2. *Aspergillus clavatus*

Colonies moderately fast growing (3.5 to 4.5 cm in 10 days at room temperature), bluish green, consisting of dense felt of conidiophores. Conidial heads clavate, large (300-400 x 150-200 µm) radiate, later splitting into several columns of compact conidial chains. Conidiophores very long, 2-4 mm in length, thin-walled, smooth, hyaline, 20-30 µm diam., gradually enlarging at the apex into clavate, club-shaped or elongate vesicles, 40-60 µm diam. Phialides in single series, covering almost the entire surface of the vesicle. Conidia smooth-walled, pale green, ellipsoidal, 3.0-5.5 x 2-3 µm.

5.2.3. *Aspergillus flavus*

Colonies are moderately fast growing (3.5-5.0 cm after 10 days at room temperature) or relatively rapid growing (6-7.5 cm after 10 days at room temperature), flat, floccose to granular, occasionally radially furrowed or cerebriform, bright yellow-green, occasionally yellow-brown with reverse cream colored or pinkish. Dark brown to black sclerotia produced in several strains, particularly in fresh isolates. Conidial heads large (300-450 µm), typically radiate, breaking into poorly defined columns with age. Conidiophores 400 to 850 µm long, thick and rough walled with subglobose or globose vesicles, 20-50 um diam. Phialides uni- or biseriate with two patterns rarely occurring in the same head, borne on the entire surface of the vesicle. Conidia round to elliptical, smooth or finely roughened, 3-6 µm diam.

5.2.4. *Aspergillus fumigatus*

Colonies spreading very fast over the medium (5.0 to 7.0 cm after 10 days at room temperature), flat, velvety or powdery to felt-like, occasionally floccose, white at first becoming green, blue green or smoky green with white margin with sporulation. Reverse cream colored, yellow, green dark or dark red-brown. Conidial heads strongly columnar and compact, often densely crowded, young conidial heads radiate. Conidiophores smooth walled, relatively short up to 300 µm long and 5-8 µm wide with a dome shaped vesicle 20-30 µm diam. Phialides uniseriate, borne only on the upper half of the vesicle with axis parallel to that of the conidiophore. Conidia green in mass, globose to subglobose, 2.5-3.5 µm diam., finely roughened.

5.2.5. **Aspergillus glaucus**

Colonies slow growing (2.5 to 3.0 cm after 10 days at room temperature), flat, powdery to densely floccose, dull green to grey-green becoming brownish green. Conidial heads, sparse, pale green, radiate, 500-1000 µm diam. Conidiophores 700-800 µm in length, hyaline, smooth-walled. Vesicles globose to club shaped. Phialides uniseriate covering the entire surface of the vesicle. Many isolates have aberrant heads with secondary conidiophores arising from the vesicle. Conidia hyaline, globose to oval, echinulate, 4.0-8.0 µm diam.

Teleomorph – *Eurotium herbariorum*. Ascoarps yellow, covered with red hyphae, spherical to subspherical, 75-200 µm diam. Asci 8-spored, 10-12 µm diam. Ascospores smooth-walled or slightly roughened with a marked equatorial furrow, hyaline or subhyaline, lenticular 6.0-7.0 x 3.0-5.0 µm.

5.2.6. ***Aspergillus nidulans***

Colonies moderately fast growing (3.5 to 4.0 cm after 10 days at room temperature), flat, velvety to powdery, green, dark-green or cream buff or honey yellow, with reverse deep red to purple. Conidiophores, brownish, 60-120 x 2-3 µm long with hemispherical vesicles, 8-10 µm diam. Phialides biseriate, borne on the upper half of the vesicle. Conidia globose to slightly elliptical, smooth-walled, subhyaline, green in mass, 3-4 µm diam.

Teleomorph - *Emericella nidulans*. Ascocarps purple, spherical, 100-200 µm, surrounded by yellowish to cinnamon brown layer of scattered hyphae bearing aggregates of pale yellow, thick-walled, spherical to subspherical Hülle cells. Asci 8-spored, spherical to subspherical, 6-12 µm. Ascospores purple, smooth-walled, lenticular with two equatorial crests, 3.5-4.5 x 3.5-4 µm (excluding crests).

5.2.7. ***Aspergillus niger***

Colonies fast growing (4.5 to 6.5 cm in 10 days at room temperature), flat, often radially furrowed, granular, initially white to yellow, becoming black with reverse cream or pale-yellow. Conidial heads large (up to 750 to 850 µm in diam.) and black, radiate, splitting into columns with age. Conidiophores very long 1.5-3.0 mm, thick-walled, smooth with large round or globose vesicle, 45-75 µm. Phialides biseriate covering the entire surface of the vesicle. Conidia brown, thick-walled, spherical or subspherical , 2.5-10 µm diam. with prominent warts and ridges.

5.2.8. ***Aspergillus ochraceus***

Colonies restricted in growth, attaining a diameter of 3.5 to 4. 0 cm after 10 days at room temperatiure, yellow to yellow-orange, ochraceus or buff, powdery to granular. Conidial heads radiate, later splitting into several columns. Conidiophores brownish, 1-1.5 m long, rough walled. Vesicles globose; phialides biseriate covering almost the entire surface of the vesicle. Conidia spherical to subspherical, 2.5-3.5 µm in diam., smooth walled to finely roughened. Pink to vinaceous-purple coloured, irregularly shaped sclerotia (up to 1 mm diam.) may be formed in some isolates.

5.2.9. ***Aspergillus oryzae***

Colonies growing rapidly (5.0 to 6.5 cm after 10 days at room temperature), pale yellow or olive yellow with different shades of green, becoming typically dull brown with age. Conidial heads radiate or loosely columnar, 100-300 µm diam. with conidial chains divergent or loosely adherent. Conidiophores very long up to 4-5 mm in length. Vesicles subspherical or occasionally flask shaped, 40 to 50 µm, occasionally reaching up to 60 to 75 µm diam. Phialides uni- or biseriate, both conditions occurring in the same strain or the same conidial head, covering the entire or three-fourths of the vesicle. Conidia greenish brown, subspherical to ovoidal, smooth-walled or roughened, 4.5-10.0 x 4.5-7.5 µm.

5.2.10. ***Aspergillus restrictus***

Colonies very restricted in growth (0.5 to 1.0 cm in diam. after 2 weeks at room temperature), velvety with central area wrinkled and raised in some strains with fimbriate margin, coloured in varying shades of green with reverse colorless or greenish. Conidial heads dark olive green, columnar, often twisted. Conidiophores hyaline, smooth-walled or finely roughened. Vesicles flask-shaped or hemispherical, 6-12 µm diam. Phialides uniseriate, covering the upper third of the vesicle. Conidia dark green in mass, cylindrical when young, becoming ellipsoidal or pyriform when mature, rough-walled, 4.0-10.0 x 3.0-6.0 µm, adherent in long columns.

5.2.11. ***Aspergillus ustus***

Colonies fast growing (4.5-6.5 cm after 10 days at room temperature), floccose, often umbonate, rarely zonate, at first white or cream yellow, soon becoming dull brown to purple gray or gray, often with purple exudate; reverse yellow, reddish or purple. Conidial heads (100-150 µm), radiate, later splitting into more or less well defined columns. Conidiophores smooth-walled, often sinuous, typically colored in brown shades with hemispherical to subspherical vesicles, 7-15 µm diam. Phialides biseriate, covering upper two-thirds of the vesicle. Conidia dark brown, spherical, rough-walled, 3.0-4.5 µm diam. Occasionally Hülle cells, often bent and twisted may be present.

5.2.12. ***Aspergillus terreus***

Colonies moderately fast rapidly growing (3.5-6.0 cm after 10 days at room temperature), flat, velvety to slightly granular, or powdery, occasionally floccose with thin irregular margins, cinnamon-buff to brown, rarely orange-brown, consisting of a dense felt of conidiophores with reverse yellow to pale brown. Amber coloured exudate produced in some strains. An isolate with deep orange colonies with lemon yellow diffusible pigment has been described. Conidial heads pale-brown, long, densely columnar, characteristically appearing fan-shaped. Conidiophores short, 100-250 µm long, flexuous, smooth-walled with dome-shaped vesicle, 10-20 µm diam. Phialides biseriate on upper two third of the vesicle. Conidia hyaline, smooth-walled, spherical to broadly elliptical, 1.5-2.5 µm diam. Round to oval, hyaline aleurioconidia with truncate base may be formed singly on the submerged hyphae in some isolates.

5.2.13. *Aspergillus versicolor*

Colonies slow growing (1.5 to 2.5 cm after 10 days at room temperature), flat, floccose, velvety or granular in shades of green, yellow, light brown or buff with considerable variation in the same culture, with reverse pale cream to red, often poorly sporulating. Conidial heads radiate, up to 100-140 µm in diam. Conidiophores, hyaline, smooth walled with subspherial or ellipsoidal vesicles. Phialides biseriate, covering the entire surface of the vesicle, sometimes reduced to *Penicillium*-like heads. Conidia spherical, echinulate, 2-3 µm diam., usually borne in loosely radiating chains. Hülle cells present in some isolates.

6. MOLECULAR APPROACHES TO CLASSIFICATION AND IDENTIFICATION OF *ASPERGILLUS* SPECIES

Molecular techniques are a powerful tool that can provide a new perspective on classification and identification of aspergilli and their detection in environment. The sequence homology within the rDNA genes of fungi (18S, 5.8S and 28S genes) and differences within the internal transcribed spacer regions (ITS1 and ITS2) are the genetic basis for organization of fungi into taxonomic groups (60). Molecular targets for the genus level identification of *Aspergillus* include the 18S rRNA gene, mitochondrial gene, and the ITS1 and ITS2. The rRNA gene for 5.8S RNA gene separates the two ITS regions. The ITS regions are located between the 18S and 28S rRNA genes and offer several advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome (61). Gaskell *et al.* (62) showed that ITS regions of the genera, *Aspergillus, Penicillium, Alternaria* and *Cladosporium* were distinct; however, they also discovered that ITS2 region alone was highly conserved among species of *Aspergillus* and *Penicillium*. Rath and Ansorg (63) found that analysis of the PCR-amplified ITS I and ITS II region by single strain conformational polymorphism (SSCP) allowed discrimination of five medically important species, viz. *A. fumigatus, A flavus, A. niger, A. terreus*, and *A. nidulans*. This method does not require morphologically fully developed colonies and yields species diagnosis faster than the conventional macroscopic and microscopic examination. In another study, Henry *et al.* (61) also showed that aspergilli could be identified from very young cultures i.e. 24 hr growth of clinical isolates by sequence comparison of both ITS1 and ITS2 regions in combination with BLAST bit score. Interspace sequence variations within multiple strains of *Aspergillus* tested were minimal in this study. Some other techniques employed earlier include use of randomly amplified DNA (RAPD) markers (64), restriction enzyme analysis of mitochondrial DNA (65), restriction fragment length polymorphism (RFLP) analysis of nuclear and mitochondrial DNA (66). Wang *et al.* (67) used 426 bp fragments of a mitochondrial (mt) cytochrome b gene amplified by polymerase chain reaction (PCR) for differentiation of *A. fumigatus, A flavus, A. niger, A. terreus*, and *Emericella* (*Aspergillus*) *nidulans* by detecting species specific nucleotides in each of these species. Geiser *et al.* (68) showed that *A. fumigatus* is close to the anamorph of *Neasartorya fischeri* by partial ß-tubulin and hydrophobin sequences. Peterson *et al.* (69) described the phylogenetic relationships of species of *Aspergillus* based on molecular studies.

Aspergillus fumigatus is the most common airborne fungal pathogen today and infections due to this fungus have become major problem in hospital hematology and transplant units. It has been necessary for epidemiological investigations to differentiate between the strains of *A. fumigatus*. In the past decade, research has concentrated on developing genotyping methods for typing of isolates of *A. fumigatus* and investigating the nosocomial origin of the infection. The techniques employed include restriction endonuclease analysis of total cellular DNA (70), ribosomal probes with part of the intergenic spacer region from the ribosomal RNA gene complex of *A. nidulans* (71). The RFLP analysis by Southern hybridization with M13 bacteriophage genome (72) gave highest level of isolate discrimination but RFLP analysis is time consuming. Southern hybridization with a retrotransposon–like element (73) has proved highly discriminatory for *A. fumigatus* strains. Bart-Delabesse *et al.* (74) tested 50 environmental, 50 clinical isolates, and 2 reference isolates with the microsatellite markers. No clustering between the environmental and clinical isolates was observed suggesting that every isolate is potentially pathogenic. Microsatellite markers may prove useful in large epidemiological studies of aspergillosis. Paris and Latge (75) reported *Afut* 2, a new family of degenerative gypsy-like retrotransposon from *A. fumigatus*, which could be used in DNA finger printing of isolates of this species. They characterized four probes from recombinant clones containing non-ribosomal repetitive sequences, which have potential for fingerprinting of isolates of *A. fumigatus*. Reiss *et al.* (76) typed approximately 1000 isolates of *A. fumigatus* from environmental and clinical sources by restriction enzyme analysis probed with an *A. fumigatus* specific retrotransposon-like sequence. These investigators found that patients with no symptoms of aspergillosis may carry several strains, whereas patients with pulmonary aspergillosis may carry one or two strains. Further it was found that environmental population of *A. fumigatus* is extremely diverse and any given environmental strain can be infectious. Nosocomial transmission of aspergillosis was proven in 39% of the patients studied. A multiple discriminatory typing procedure is required to understand the ecology of *A. fumigatus* strains and epidemiology of infections due to this fungus.

7. GENETICS OF *ASPERGILLUS*

Classical genetics of several pathogenic species of *Aspergillus* including *A. fumigatus*, *A. niger*, and *A. nidulans* has been studied. *Aspergillus nidulans* has been extensively studied for conventional genetic analysis by various investigators because of its both asexual and sexual life cycles. A considerable amount of information on genomic structure and gene regulation of this organism is available. The genetics of population, conidiation, regulation of carbon and nitrogen metabolism in *Aspergillus* has been very well described by Smith and Pateman (77). During the past few decades, molecular

techniques have been applied to isolate and clone structural, regulatory genes and also genes responsible for morphogenetic processes and several metabolic functions (78).

Genes encoding for multiple drug resistance have been identified in some *Aspergillus* species, such as *A. fumigatus* and *A. flavus* (79). Very recently Edlind *et al.* (80) have cloned and sequenced the VYP51 gene, which encodes the target of antifungal azoles, namely cytochrome P450 sterol 14, α-demethylase. Rhodes *et al.* (81) have identified the genes encoding the regulatory subunit of cyclic adenosine monophosphate (cAMP)-dependent protein kinase and a member of *ras* gene family in *A. fumigatus*, both of which are involved in cAMP-mediated signaling in fungi. These genes were found to regulate growth of the fungus on endothelial cells in murine model. Boettner *et al.* (82) were able to clone CgrA, the ortholog of the yeast nucleolar protein Cgr1p. Orthologs of the *A. fumigatus* Cgr1 protein have also been cloned from *A. nidulans* (83) and *Saccharomyces cerevisiae* (84). It is speculated that targeting nucleolar proteins involved in ribosome biogenesis would be an effective therapeutic strategy against infections due to *Aspergillus* and other fungi. The cloning of the *A. fumigatus* CgrA gene is the first important step to this goal (82). There have also been some studies on gene disruption to identify virulence factors. The gene pyrG, which encodes an enzyme required for *de novo* synthesis of uracil, *alb1* (*pksp*), which enables a polyketide synthase involved in conidial pigment production, *chs*G, which encodes a class III chitin synthase, and *are*A, a transcription factor regulating nitrogen metabolism, have been shown to reduce virulence of a deletion mutant in a mouse model (85-89). Tang *et al.* (90) reported that lysine and aminobenzoic acid (PABA) deficiency in *A. nidulans* was associated with avirulence. Likewise Sandhu *et al.* (91) established that PABA deficiency in auxotrophic mutants of *A. fumigatus* resulted in loss of virulence.

8. CONCLUSION

The genus *Aspergillus* has about 180 accepted species. The circumscription and classification of these species has been based primarily on difference of their morphological and cultural characteristics. The complexity of the genus is emphasized by the number and diversity of associated teleomorphs (10 in Trichocomaceae). About 40 species have been isolated from humans. Though the identification of *Aspergillus* species based on traditional morphological criteria is very effective, the large number of species emphasizes the use of alternative approaches. Several studies based on combinations of different types of morphological, biochemical and physiological characteristics have been used but their practical utility is yet to be established. Molecular techniques though widely applied are still in a developmental phase due to insufficient availability of reference data. Further the degree of variability of most of the species for the particular gene is unknown (18). Analysis of rRNA sequences has proved useful in identification of certain strains of *Aspergillus* species, which are difficult to identify by morphological criteria (14, 34). Identification of pathogenic species of *Aspergillus* from 24 hr old cultures using nucleic acid sequential analysis of ITS1 and ITS2 regions in combination with BLAST bit score is a reliable and efficient method that provides earlier diagnosis than standard culture methods (61). The use of ITS sequencing should be further explored for identification of *Aspergillus* species in culture and directly in clinical specimens. Further research on molecular biology and genetics of *A. fumigatus* is needed to develop accurate genotyping methods for clinical and environmental isolates of this species for a better understanding of epidemiology and pathogenesis of infections caused by it.

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