Global molecular epidemiology and genetic diversity of Fusarium, a significant emerging group of human opportunists from 1958 to 2015

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**INTRODUCTION**

Fusarium infections are a major challenge with respect to the diagnosis and treatment, especially in neutropenic patients. Disseminated infections may be fatal and are a considerable source of increased healthcare costs. A major area of concern is the intrinsic resistance to a broad range of antifungals,1 which is a characteristic of Fusarium. During the past decade, the F. solani complex has received special interest because of the increasing numbers of infections worldwide.2 More than 300 cases of Fusarium keratitis were associated with contaminated contact lens cleaning solution, causing outbreaks between 2005 and 2007, where members of the F. solani species complex played a major role.3 Furthermore, reservoirs of infectious Fusarium species in hospital environments, especially plumbing and water systems, have been reported.4

Although human fusariosis was only recognized since the late 1950s and endemic areas are mostly located in tropical and subtropical countries,5 their global significance has only recently come into focus within the past three decades. Etiological agents differ in antifungal susceptibility,6 virulence profiles, geographic distribution, ecological niches, life cycle, host and mycotoxin production.7 Although agents of fusariosis are mostly environmental,8 the potential of nosocomial transmission has recently been raised,9 especially with reference to the high mortality rate of ~90% in patients with prolonged and severe neutropenia.10

The burden of disease has not been established, but numerous clinical case series and case reports provide an estimate of the magnitude of the problem. Most published studies have focused on prevalence in single healthcare centers.10–16 Nucci et al.17 reported 233 cases from different hospitals on a global scale. Mohammed et al.18 reported 26 cases from the United States and reviewed 97 cases from the literature, and Horn et al.12 described 65 cases from the North American Path Alliance Registry. A major problem in comparative studies is the subdivision of the classical species into a series of molecular siblings, which renders the older literature without sequence data uninterpretable. Despite the current clinical importance of the organism, the phylogenetic relationships among species, varieties and geographical groups in Fusarium are currently elusive. Hence, the re-interpretation of these data in the light of modern molecular phylogeny is compulsory.

Molecular phylogenetic studies have led to the description of many Fusarium species with clinical relevance. These include members of the F. solani species complex, namely, F. falciforme, F. keratoplasticum, F. oxysporum, and F. fujikuroi.
F. lichenicola, F. petroliophilum, F. pseudosieriforme and F. solani (FSSC5), which is also known as Fusisporium solani and Fusarium haplotype ‘6’. The F. oxysporum species complex (FOSC) contains three lineages, which are involved in fusariosis and still have not been formally introduced as taxonomic species. The F. fujikuroi species complex includes F. acutatum, F. ananatum, F. anthophilum, F. anidyaizi, F. fujikuroi s.s., F. globosum, F. guttiforme, F. musae, F. napsiforme, F. nygamai, F. verticillioides, F. proliferatum, F. ramarum, F. sacchari, F. subglutinosus, F. temperatum and F. thapsinum. Although rare, species of other Fusarium lineages are emerging as potential opportunistic pathogens, for example, in the F. incarnatum-equiseti species complex (FIESC; F. incarnatum and F. equiseti), the F. dimerum species complex (F. dimerum, F. delphinioides and F. penzei). The F. chlamydosporum species complex, the F. sambucinum species complex (F. armeniacum, F. brachygibbosum, F. langetheiae and F. sporotrichioides) and the F. tricinctum species complex (F. acuminatum and F. flaccifermum). Over the past decade, the number of cases of fusariosis has increased worldwide, but there are only a few reports describing the molecular epidemiology; therefore, the aim of the present study is to introduce a hypothetical system that permits the interpretation and use of at least a part of the literature where sequence data are lacking. Pre-molecular publications, which include interpretable case reports and geographical information, were collected. Subsequently, available Fusarium strains that were collected worldwide and deposited during the past century in the CBS-KNAW, Fungal Biodiversity Centre, culture collection Utrecht, The Netherlands, were sequenced and re-identified with current diagnostic technology, which enables the phylogenetic analysis of the human–pathogenic Fusarium species. These data were then compared with published materials and their distribution with the assumption that their distributions in each region had remained unaltered.

MATERIALS AND METHODS

Fungal strains

A total of 127 strains collected from clinical samples (n = 74; 58.3%; collected between 1978 and 2015) and strains collected from the environment (n = 53; 41.7%; collected between 1929 and 2015) were analyzed. All of the strains were maintained under the name ‘Fusarium’ in the reference collection of CBS-KNAW, Utrecht, the Netherlands. The data regarding geographic origins and sources of isolation are listed in Table 1. All of the available type strains were included. Stock cultures were maintained on slants of 2% malt extract agar at 24 °C. The strains were assigned to a clinical subgroup and an environment subgroup.

DNA extraction

DNA was extracted following the Quick Cetyl trimethylammonium bromide (CTAB) protocol. A total of 1–10 mm³ fungal material was transferred to 2 mL screw-capped tubes prefilled with 490 μL of 2 × CTAB buffer and 6–10 acid-washed glass beads. A total of 10 μL of proteinase K was added and mixed thoroughly on a MoBio vortex (MO BIO Laboratories, Inc., Carlsbad, CA, USA) for 10 min. Then, 500 μL of chloroform:isoamylalcohol (24:1) was added and shaken for 2 min after incubation for 60 min at 60 °C. The tubes were centrifuged for 10 min at 14 000 rpm, and the supernatant was collected in a new Eppendorf tube. To ~400 μL of the DNA sample, 2/3 vol (~270 μL) of ice-cold isopropanol was added and centrifuged again at 14 000 rpm for 10 min, and the upper layer was dissolved in 1 mL ice-cold 70% ethanol. The tubes were centrifuged again at 14 000 rpm for 2 min, air-dried and resuspended in 50 μL TE buffer. The quality of the genomic DNA was verified by running 2–3 μL on a 0.8% agarose gel. Then, the DNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher, Wilmington, DE, USA), and the samples were stored at −20 °C until ready for analysis.

DNA amplification and sequencing

The following two gene regions were amplified directly from the genomic DNA: the second largest subunit of RNA polymerase (RPB2; Reeb et al.19) and the translation elongation factor-1α (TEF1α; O’Donnell et al.20) were amplified and sequenced following the methods published by Saleh et al.16 The PCR reactions were performed in a volume of 12.5 μL containing 1.25 μL of 10× PCR buffer, 7.5 μL of water, 0.5 μL of dNTP mix (2.5 mM), 0.25 μL of each primer (10 pmol), 0.05 μL of Taq polymerase (5 U/μL), 0.7 μL of dimethylsulphoxide and 1 μL of template DNA (100 ng/μL). The amplification was performed with the ABI Prism 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). The cycling conditions included 1 cycle of 5 min at 94 °C, 10 cycles of 45 s at 94 °C, 45 s at 55 °C and 1.5 min at 72 °C, 30 cycles of 45 s at 94 °C, 45 s at 52 °C and 1.30 min at 72 °C; a post elongation step of 6 min at 72 °C for TEF1 (EF1, EF2) and a pre-denaturation for 3 min at 95 °C, 5 cycles of 45 s at 95 °C, 45 s at 58 °C and 2 min at 72 °C, 5 cycles of 45 s at 95 °C, 45 s at 56 °C and 2 min at 72 °C, 30 cycles of 45 s at 95 °C, 45 s at 52 °C and 2 min at 72 °C, and a post elongation step of 8 min at 72 °C for RPB2 (SF2 and 7cR). The PCR products were visualized by electrophoresis on 1% (w/v) agarose gels. The sequencing PCR was performed as follows: 1 min at 95 °C followed by 30 cycles consisting of 10 s at 95 °C, 5 s at 50 °C and 2 min 60 °C. The reactions were purified with Sephadex G-50 fine (GE Healthcare Bio-Sciences, Uppsala, Sweden), and the sequencing was conducted on an ABI 3730xl automatic sequencer (Applied Biosystems) with a BigDye v3.1 terminator cycle sequencing kit (Applied Biosystems).

Identification

The strains were identified by BLAST in GenBank, Fusarium MLST (http://www.cbs.knaw.nl/fusarium/)20 and the FUSARIUM-ID (http://isolate.fusariumdb.org/)21 databases. In addition, the phylogenetic placements with species/haplotypes within species complexes were verified with available databases that are specific for Fusarium.

Phylogenetic analyses

Sequences of TEF1 and RPB2 were undertaken to extend the genetic characterization of 127 isolates of Fusarium species previously characterized in terms of morphological characteristics. The sequences were edited using SeqMan in the Lasergene package (DNAstar, Madison, WI, USA). A phylogenetic approach was used to investigate the relationship between 65 strains of Fusarium species including type and reference strains. The sequences were aligned using MAFFT v. 7.127 (http://mafft.cbrc.jp) followed by manual adjustments with MEGA v. 6.2.22 A combined alignment was constructed for RPB2 and TEF1 for both the reference and test strains. The best-fit model of evolution was determined by MEGA v. 6.2.22 A bootstrapped maximum-likelihood analysis was performed using RAXMLVI-HPC v. 7.0.323 as implemented on the Cipres portal (http://www.phylo.org/),24 with non-parametric bootstrapping using 1000 replicates. Detailed analyses of medically important strains were compared in relation with their clinical cases. For instance, F. solani actually represents a complex (that is, the F. solani species complex).
| CBS number | Species name         | Country         | Source                       | GenBank accession number |
|------------|----------------------|-----------------|------------------------------|--------------------------|
| CBS 130548 | F. acutatum          | Iran            | Onychomycosis (Human)        | KR071756                 |
| CBS 113964 | F. acutatum          | Egypt           | Environmental                | KR071759                 |
| CBS 739.97 | F. acutatum          | India           | Environmental                | KR071757                 |
| CBS 401.97 | F. acutatum          | India           | Environmental                | KR071755                 |
| CBS 402.97 | F. acutatum          | India           | Environmental                | KR071754                 |
| CBS 118517 | F. ananatum          | South Africa    | Environmental                | KR071761                 |
| CBS 118518 | F. ananatum          | South Africa    | Environmental                | KU711690                 |
| CBS 118516 | F. ananatum          | South Africa    | Environmental                | KU711680                 |
| CBS 184.29 | F. ananatum          | Unknown         | Environmental                | KR071762                 |
| CBS 256.93 | F. andiyazi          | Cuba            | Environmental                | KR071719                 |
| CBS 119857 | F. andiyazi          | South Africa    | Environmental                | KP662901                 |
| CBS 737.97 | F. anthropophilum    | Germany         | Environmental                | KU711685                 |
| CBS 222.76 | F. anthropophilum    | Germany         | Environmental                | KU711685                 |
| CBS 118585 | F. anthropophilum    | USA             | Environmental                | KR071764                 |
| CBS 119859 | F. anthropophilum    | New Zealand     | Environmental                | KR071765                 |
| CBS 961.87 | F. concolor          | South Africa    | Environmental                | KR071773                 |
| CBS 676.94 | F. concolor          | South Africa    | Environmental                | KR071774                 |
| CBS 111770 | F. concolor          | Spain           | Keratitis (Human)            | KU711719                 |
| CBS 111770 | F. delphinooides     | India           | Keratitis (Human)            | KU711775                 |
| CBS 135550 | F. dimerum           | Mexico          | Keratitis (Human)            | KU711721                 |
| CBS 135552 | F. equiseti          | Mexico          | Keratitis (Human)            | KU711723                 |
| CBS 135553 | F. equiseti          | Mexico          | Keratitis (Human)            | KU711722                 |
| CBS 135532 | F. falciforme        | Mexico          | Keratitis (Human)            | KU711737                 |
| CBS 135533 | F. falciforme        | Mexico          | Keratitis (Human)            | KU711738                 |
| CBS 135521 | F. falciforme        | Mexico          | Keratitis (Human)            | KU711733                 |
| CBS 135520 | F. falciforme        | Mexico          | Keratitis (Human)            | KU711732                 |
| CBS 135526 | F. falciforme        | Mexico          | Keratitis (Human)            | KU711734                 |
| CBS 135524 | F. falciforme        | Mexico          | Keratitis (Human)            | KU717351                 |
| CBS 135558 | F. falciforme        | Mexico          | Keratitis (Human)            | KU717356                 |
| CBS 135559 | F. falciforme        | Mexico          | Keratitis (Human)            | KU717357                 |
| CBS 135513 | F. falciforme        | Mexico          | Keratitis (Human)            | KU717245                 |
| CBS 135512 | F. falciforme        | Mexico          | Keratitis (Human)            | KU717245                 |
| CBS 135522 | F. falciforme        | Mexico          | Keratitis (Human)            | KU717255                 |
| CBS 135523 | F. falciforme        | Mexico          | Keratitis (Human)            | KU717265                 |
| CBS 135528 | F. ficicrescens      | Iran            | Environmental                | KP662898                 |
| CBS 125177 | F. ficicrescens      | Iran            | Environmental                | KP662899                 |
| CBS 125178 | F. ficicrescens      | Iran            | Environmental                | KP662900                 |
| CBS 125181 | F. ficicrescens      | Iran            | Environmental                | KP662900                 |
| CBS 449.95 | F. fujikuroi         | France          | Environmental                | KR071742                 |
| CBS 257.52 | F. fujikuroi         | Japan           | Environmental                | KU711678                 |
| CBS 262.54 | F. fujikuroi         | India           | Environmental                | KR071744                 |
| CBS 221.76 | F. fujikuroi         | Taiwan          | Environmental                | KR071741                 |
| CBS 130402 | F. fujikuroi         | USA             | Human skin (Human)           | KU711677                 |
| CBS 121864 | F. fujikuroi         | USA             | Environmental                | KR071743                 |
| CBS 119855 | F. fujikuroi         | USA             | Environmental                | KU711679                 |
| CBS 454.97 | Fusarium sp          | Sudan           | Environmental                | KU711697                 |
| CBS 483.94 | Fusarium sp          | Australia       | Environmental                | KU711698                 |
| CBS 119850 | Fusarium sp          | Australia       | Environmental                | KU711699                 |
| CBS 135528 | F. keratoplasticum   | Mexico          | Keratitis (Human)            | KU717432                 |
| CBS 135528 | F. keratoplasticum   | Mexico          | Keratitis (Human)            | KU717432                 |
| CBS 135527 | F. keratoplasticum   | Mexico          | Keratitis (Human)            | KU717432                 |
| CBS 135531 | F. keratoplasticum   | Mexico          | Eumycetoma (Human)           | KU717432                 |
| CBS 135530 | F. keratoplasticum   | Mexico          | Eumycetoma (Human)           | KU717432                 |
| CBS 135529 | F. keratoplasticum   | Mexico          | Keratitis (Human)            | KU717432                 |
| CBS number | Species name | Country | Source | GenBank accession number |
|------------|--------------|---------|--------|-------------------------|
| dH21918/F605 | F. keratoplasticum | Netherlands | Nail infection (Human) | KU711746 KU604344 |
| dH22043/F609 | F. keratoplasticum | Netherlands | Foot infection (Human) | KU711747 KU604341 |
| CBS 748.97 | F. napiforme | Namibia | Environmental | KU711748 KU604346 |
| CBS 674.94 | F. napiforme | Australia | Environmental | KU711749 KU604347 |
| CBS 135139 | F. napiforme | India | Keratitis (Human) | KU711750 KU604348 |
| CBS 135140 | F. napiforme | India | Keratitis (Human) | KU711751 KU604349 |
| dH 21772/F602 | F. oxysporum | Netherlands | Nail infection (Human) | KU711752 KU604350 |
| dH22047/F611 | F. oxysporum | Netherlands | Nail infection (Human) | KU711753 KU604351 |
| CBS 135560 | F. oxysporum | Mexico | Keratitis (Human) | KU711754 KU604352 |
| CBS 135561 | F. oxysporum | Mexico | Keratitis (Human) | KU711755 KU604353 |
| CBS 463.91 | F. oxysporum | Germany | Nail infections (Human) | KU711756 KU604354 |
| CBS 135515 | F. petroliphilum | Mexico | Keratitis (Human) | KU711757 KU604355 |
| CBS 135518 | F. petroliphilum | Mexico | Keratitis (Human) | KU711758 KU604356 |
| CBS 135519 | F. petroliphilum | Mexico | Keratitis (Human) | KU711759 KU604357 |
| CBS 135535 | F. petroliphilum | Mexico | Keratitis (Human) | KU711760 KU604358 |
| CBS 135514 | F. petroliphilum | Mexico | Mycotic keratitis (Human) | KU711761 KU604359 |
| CBS 187.34 | F. phyllophorum | UK | Environmental | KU711762 KU604360 |
| CBS 246.61 | F. phyllophorum | USA | Environmental | KU711763 KU604361 |
| CBS 480.77 | F. proliferatum | Netherlands | Environmental | KU711764 KU604362 |
| CBS 182.32 | F. proliferatum | USA | Environmental | KU711765 KU604363 |
| CBS 183.29 | F. proliferatum | Japan | Environmental | KU711766 KU604364 |
| CBS 184.33 | F. proliferatum | Guyana | Environmental | KU711767 KU604365 |
| CBS 125014 | F. proliferatum | USA | Deep infection (Human) | KU711768 KU604366 |
| CBS 131391 | F. proliferatum | Australia | Environmental | KU711769 KU604367 |
| CBS 133030 | F. proliferatum | Iran | Onycomycosis (Human) | KU711770 KU604368 |
| CBS 135547 | F. proliferatum | Mexico | Keratitis (Human) | KU711771 KU604369 |
| CBS 135549 | F. proliferatum | Mexico | Keratitis (Human) | KU711772 KU604370 |
| CBS 116324 | F. proliferatum | Spain | Keratitis (Human) | KU711773 KU604371 |
| CBS 130179 | F. proliferatum | USA | Deep infection (Human) | KU711774 KU604372 |
| CBS 135142 | F. sacchari | India | Corneal ulcer (Human) | KU711775 KU604373 |
| CBS 135143 | F. sacchari | India | Corneal ulcer (Human) | KU711776 KU604374 |
| CBS 135144 | F. sacchari | India | Corneal ulcer (Human) | KU711777 KU604375 |
| CBS 135145 | F. sacchari | India | Corneal ulcer (Human) | KU711778 KU604376 |
| CBS 223.76 | F. sacchari | India | Environmental | KU711779 KU604377 |
| CBS 134.73 | F. sacchari | Guyana | Environmental | KU711780 KU604378 |
| CBS 131369 | F. sacchari | Australia | Environmental | KU711781 KU604379 |
| CBS 121683 | F. sacchari | India | Endophthalmitis (Human) | KU711782 KU604380 |
| CBS 135563 | F. solani (FSSC5) | Mexico | Hyalohyphomycosis (Human) | KU711783 KU604381 |
| CBS 135564 | F. solani (FSSC5) | Mexico | Hyalohyphomycosis (Human) | KU711784 KU604382 |
| CBS 135565 | F. solani (FSSC5) | Mexico | Hyalohyphomycosis (Human) | KU711785 KU604383 |
| CBS 119831 | F. subglutinans | New Guinea | Environmental | KU711786 KU604384 |
| CBS 747.97 | F. subglutinans | USA | Environmental | KU711787 KU604385 |
| CBS 135538 | F. temperatum | Mexico | Pulmonary infection (Human) | KU711788 KU604386 |
| CBS 135539 | F. temperatum | Mexico | Pulmonary infection (Human) | KU711789 KU604387 |
| CBS 135540 | F. temperatum | Mexico | Keratitis (Human) | KU711790 KU604388 |
| CBS 135541 | F. temperatum | Mexico | Keratitis (Human) | KU711791 KU604389 |
| CBS 776.96 | F. hispinum | USA | Environmental | KU711792 KU604390 |
| CBS 733.97 | F. hispinum | South Africa | Environmental | KU711793 KU604391 |
| CBS 130176 | F. hispinum | Italy | Human mycetoma (Human) | KU711794 KU604392 |
| CBS 119833 | F. hispinum | USA | Environmental | KU711795 KU604393 |
| CBS 109077 | F. hispinum | Ethiopia | Environmental | KU711796 KU604394 |
AFLP
The *Fusarium* strains were subjected to amplified fragment length polymorphism (AFLP) genotyping using a previously described method. However, for the amplification of the DNA fragments, the selective residues (underlined) of the HpyCH4IV-primer (5’-GAT GAG TCC TGA CTA ATG AG-3’) and MseI-primer (5’-Flu-GTA GAC TGC GTA CCC GTAC-3’; MseI-C-selective primer) were replaced. The amplicons were diluted 20 × with double-distilled \( H_2O \); 1 μL of the diluted amplicon was then added to a mixture of 8.9 μL \( ddH_2O \) and 0.1 μL LIZ600 (Applied Biosystems) followed by a heating step for 1 min at 100 °C and cooling to 4 °C. The AFLP fragment analysis was conducted using an ABI3500xL Genetic Analyzer (Applied Biosystems) according to the manufacturer’s instructions. The raw data were then inspected visually after importation into BioNumerics v7.5 (Applied Maths, Sint Martens-Latem, Belgium) and analyzed by an Unweighted Pair Group Method with Arithmetic Mean clustering using the Pearson correlation coefficient. The analysis was restricted to DNA fragments in the range of 40–400 bp. The final AFLP dendrograms were based on the combination of sequencing and the AFLP data of both dendrograms.

Meta-analysis
The authors analyzed the existing medical literature on human cases of fusariosis from 1958 until December 2015. The authors conducted a systematic literature search using PubMed, and the terms *Fusarium* and 'fusariosis' were used for the search and both were also used as MeSH words and free words. Studies were only included that reported data for the individual cases because data provided in aggregate often lacked specific information for individual cases. Only cases with either histologically or culturally proven *Fusarium* infection were included. A total of 388 case reports in ~ 265 published studies were collected on a worldwide basis. The numbers are approximate because some cases have been used in repeated publications. Only cases with either histologically or culturally proven *Fusarium* infection were included (Supplementary Reference S1).

RESULTS
Types of articles
A total of 388 cases of fusariosis from 1958 until December 2015 were used in the literature data analysis. This included articles that were mostly single case reports, two patient cases and a series of cases of fusariosis. The reported cases of fusariosis were identified from all over the world, and particularly from tropical and subtropical countries with a large agrarian population such as Brazil, China, Colombia, India and Mexico. The other areas with frequent fusariosis were Australia, South Africa, Turkey and the Americas. *Fusarium* infections have also been reported from different countries in eastern and western Europe.

Patient characteristics. An overview of the cases of fusariosis published in the medical literature, which includes the great majority of cases published to date, is provided in Table 2. The majority of patients were male (\( n = 253; 65.2\% \); mean 41 years; range three months–83 years). Over a third of the patients (\( n = 143; 36.9\% \)) had various underlying conditions at the time when the *Fusarium* infection was diagnosed. Causes of immunosuppression were hematological diseases and hematologic malignancies (\( n = 122; 31.4\% \)) and cancer of the solid organs (\( n = 17; 4.8\% \)). Other causes of immunosuppression were medication (\( n = 140; 36\% \)), which included antibiotic (\( n = 34; 8.8\% \)) and steroid treatment (\( n = 10; 2.6\% \)). Pathogen introduction was ranked as trauma (\( n = 18; 4.6\% \)), indwelling catheters (\( n = 2; 0.5\% \)), nasogastric tubes (\( n = 2; 0.5\% \)) and dialysis (\( n = 3; 0.77\% \)). No metabolic disorders, such as diabetes, were recorded in association with infection.

Type of infections
Infections due to *Fusarium* were predominantly found to be superficial and subcutaneous (\( n = 174; 44.8\% \)), occurring on the skin (\( n = 62; 16\% \)), eyes (\( n = 66; 17\% \)) and nails (\( n = 25; 6.4\% \)). Deep infections involved bone, joint and lung (\( n = 4; 1\% \)), heart (\( n = 3; 0.77\% \)), and peritoneum (\( n = 2; 0.5\% \)). The sum of the invasive and disseminated cases was \( n = 109 (28\% \)), some of which were associated with fungemia (\( n = 25; 6.4\% \)) or disseminated disease with brain abscesses (\( n = 4; 1\% \); Table 2).

Treatment
An overview of the reported treatment of the cases of fusariosis is shown in Table 3. The most widely used antifungal agent was amphotericin B deoxycholate (\( n = 198; 51\% \)), followed by liposomal amphotericin B (\( n = 45; 11.6\% \)), voriconazole (\( n = 42; 10.8\% \)),...
Table 2 Characteristics of 388 patients with fusariosis and literature cases from 1958 until 2015

| Characteristic               | Number of patients |
|------------------------------|--------------------|
| Total                        | 388                |
| Age, years (range)           | 3 months – 82 years |
| Sex, M:F:unknown             | 253 (65.3%): 125 (32.2%): 10 (2.5%) |

Underlying condition

| Transplantation             |                |
|-----------------------------|----------------|
| Liver                       | 5 (1.2%)       |
| Lung                        | 4 (1%)         |
| Bone marrow                 | 29 (7.5%)      |
| Multivisceral               | 1 (0.25%)      |
| Kidney                      | 3 (0.77%)      |
| Heart                       | 4 (1%)         |
| Stem cells                  | 38 (9.8%)      |

Trauma/burns                  | 27 (7%)        |

Foreign body                  | 18 (4.6%)      |

Contact lens                  | 4 (1%)         |

Catheter                     | 2 (0.5%)       |

Graft                        | 3 (0.77%)      |

Nasogastric tube             | 3 (0.77%)      |

Dialysis                     | 4 (1%)         |

Cancer                       |                |

Hematologic                  | 122 (31.4%)    |

Solid organ                  | 17 (4.8%)      |

Medication                   |                |

Antibiotics                  | 140 (36%)      |

Steroids                     | 34 (8.8%)      |

No                           | 20 (5%)        |

Site of infection

Superficial                  |                |

Skin                         | 62 (16%)       |

Eye                          | 66 (17%)       |

Nail                         | 25 (6.44)      |

Bone                         | 4 (1%)         |

Joint                        | 4 (1%)         |

Lung                         | 4 (1%)         |

Endocarditis                 | 3 (0.77%)      |

Peritoneum                   | 2 (0.5%)       |

Perinephric abscess          | 2 (0.5%)       |

Disseminated                 | 109 (28%)      |

Blood                        | 25 (6.4%)      |

Brain                        | 4 (1%)         |

Abbreviations: female, F; male, M.

5-flucytosine (n = 30; 7.7%), itraconazole (n = 26; 6.7%), fluconazole (n = 25; 6.4%) and ketoconazole (n = 19; 4.9%).

The antifungal combinations used in treating fusariosis were given either as a two- or a three-drug combination. The most frequently used combination of two drugs was amphotericin B with voriconazole (n = 24; 6%), followed by amphotericin B with 5-flucytosine (n = 20; 5%), amphotericin B with ketoconazole (n = 4; 1%) and amphotericin B with fluconazole (n = 4; 1%). Other combinations were used in one or two cases. Triple combinations were used in 14 cases (n = 14; 3.6%). In addition, surgery with antifungal treatment was used in 80 cases (20.6%). In addition to antifungal therapy and surgery, granulocyte transfusions or granulocyte–colony-stimulating factor transfusions were also used. Only seven isolates were associated with cases where no treatment was reported (Table 3). It was not possible to look at the changes in treatment over time, although the authors assume that azole treatments have increased while AmB has declined. With the current guidelines, liposomal amphotericin B (n = 45; 11.6%) and voriconazole (n = 42; 10.8%) are very similar according to the data from the reported cases.

Genetic analysis

A total of 127 Fusarium strains deposited in the CBS-KNAW collection were partially sequenced for RPB2 and TEF1. The resulting two phylogenies yielded almost identical topologies with similar
resolution. Almost all of the strains of known species in all complexes of Fusarium formed independent clades in each tree. A concatenated tree (Figure 1), including all major human–pathogenic complexes of Fusarium, was based on 146 selected sequences. The lengths of the generated sequence data were 795 and 507 bp for RPB2 and TEF1, respectively. Of the 1302 nucleotides sequenced, 720 (55.1%) were constant, 551 (42.2%) were parsimony informative and 576 (44.1%) were variably and parsimony non-informative using MEGA v. 6.2.22

The combined tree was subdivided into several species complexes with high bootstrap values (Figure 1). Seven clades represented human opportunists within the F. solani species complex. Thirteen groups represented opportunistic species in the F. fujikuroi species complex with smaller human-associated clusters in the FOSC and to a lesser extent in the F. chlamydosporum, F. polyphialidicum (syn. F. concolor), F. dimerum and F. incarnatum species complexes. Strains CBS 454.97, CBS 483.94 and CBS 119850 were identified morphologically as F. napiforme but formed a separate cluster that was different from the three strains including the type strain of F. napiforme (Figure 1).

The AFLP profiles contained ~ 50 – 60 fragments in the range of 40 – 400 bp. The AFLP dendrogram comprised seven main clusters at the species complex level and additional subgroups within the main species clusters revealed genetic diversity within each species complex (Figure 2). However, the profiles did not significantly vary between the F. solani species complexes, such as F. falciforme, F. keratoplasticum, F. lichenicola F. petroliphilum and F. pseudensiforme, whereas there was significant AFLP variation between isolates within the F. fujikuroi species complex with separate profiles for each species and within other species complexes of F. chlamydosporum, F. concolor, F. dimerum, F. incarnatum-equiseti and F. oxysporum.

When comparing the AFLP clusters with the distribution of DNA sequence lineages, the groups were largely concordant. Groups 1 – 7 matched with previous identifications using RPB2 and TEF1 sequences. The Fusarium concolor species complex had one clinical subgroup, the F. dimerum species complex had two and the Fusarium fujikuroi species complex consisted of 16 clinical subgroups (15 named subgroups and 1 unnamed molecular lineage). The FIESC had a single clinical group, the FOSC was divided into two subgroups and the F. solani species complex comprised six named and one unnamed subgroup. The AFLP clusters and subclusters were almost identical to the sequencing identifications except for few strains within the

![Figure 1](image-url) A phylogenetic tree resulting from the RAxML analysis for the RPB2 and TEF1 genes. The total alignment length is 1302 bp. A maximum-likelihood analysis was performed using RAxML with non-parametric bootstrapping using 1000 replicates. The numbers above the branches are bootstrap support values ≥ 0.70. The outgroup was the epitype (ET) strain of F. dimerum CBS 108944.
Figure 2  Clustering of the amplified fragment length polymorphism banding pattern of *Fusarium* spp. combined with a sequence analysis of *RPB2* and *TEF1* constructed by Bionumerics v7.5 (Applied Maths). The dendrogram was generated using the Unweighted Pair Group Method with Arithmetic Mean algorithm.

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of human Fusarium infections.10–13,27–34 The 127 Fusarium strains from the current study were collected from 26 countries in six continents and included clinical and environmental strains and isolates from cold blooded animals. Of these, Australia, Brazil, India, Mexico and the USA were among the top 10 countries with the highest Fusarium infections based on clinical isolates in the CBS collection. Not surprisingly, 75 of the 127 patients from this study acquired their infection in one of these countries. Although human opportunists were highlighted in many studies focusing on specific regions of the world and specific types of infections,10–13,27–34 the 127 Fusarium strains from the current study were collected from 26 countries in six continents and included clinical and environmental strains and isolates from cold blooded animals. Of these, Australia, Brazil, India, Mexico and the USA were among the top 10 countries with the highest Fusarium infections based on clinical isolates in the CBS collection. Not surprisingly, 75 of the 127 patients from this study acquired their infection in one of these countries.

Previously, the majority of the clinically relevant Fusarium species were classified as two species complexes that in the past were referred to as a single species, *F. oxysporum* and *F. solani*.35 Approximately 80% of human infections are caused by members of both species complexes,36 but a significant share of infections is caused by the following novel species complex members: *F. dimerum*, *F. falkumori* and *F. incarnatum-equiseti*. Within the *F. solani* complex, there are six recognized species and one unnamed lineage (FSSC6) clinically involved in fusariosis (Figure 1). Of these species, *F. falciforme* (*n* = 14/127 cases; 11%) was the dominant species in our study and mainly isolated from keratitis cases in Brazil, India and Mexico. Recently, Hassan et al.13 showed that the majority of keratitis cases (*n* = 46/65 cases; 70.7%) were *F. falciforme*. This species is emerging as one of the most virulent Fusarium species associated with fusariosis and keratitis.15,36,37

In the 2005–2006 mycotic keratitis outbreaks in Southeast Asia and North America that were associated with a contact lens cleaning solution, *F. petrophilum* and *F. keratoplasticum* were the most common species,36 which is consistent with the current study. The AFLP genotypic variability was higher in the environmental species than in the clinical species. A potential explanation is that not all environmental genotypes are sufficiently adapted to the host tissue and are not selected or perhaps a sampling effect is involved. Zhang et al.35 studied the *F. solani* species complex, specifically those species that cause infections in humans and plants, and concluded that clinical isolates often shared multi-locus haplotypes with isolates from different environmental sources, including hospital locations. An increase of fusariosis among immunosuppressed patients was noted in the bone marrow transplant unit and among patients with superficial infections in a hospital in Rio de Janeiro, Brazil.38 These authors concluded that this increase might be due to airborne conidia circulating in this geographical region. Short et al.36 concluded that there is no evidence that clinical isolates differ from those collected from other sources.

The large diversity of the FOSC is not completely resolved, and it is not yet known whether the species have one or several phylogenetic origins or whether a single species or a species complex is concerned. From a traditional taxonomic point of view, *F. oxysporum* isolates are differentiated from each other based on the pathogenicity as *formae speciales*, but this has been shown to be an unreliable approach.8 In addition, the species delimitation was for the FOSC, and at least 26 sequence types within the complex were involved in human infections.39 Our FOSC clinical isolates were distributed throughout the complex, although some clustering was found in the clade marked ‘sequence type 33’, which is based on TEF1 alone, and this sequence type is considered the most common clade that contains clinical *F. oxysporum* strains. The remaining species complexes of *F. chlamydosporum*, *F. concolor*, *F. dimerum* and *F. incarnatum-equiseti* form separate clusters in the highly resolved sequence-based maximum likelihood tree (Figure 1).

The FIESC compromises 28 phylogenetically distinct lineages,34 and only 2 are named and mainly involved in human infections (*F. incarnatum* and *F. equiseti*).40 Although several members of the FIESC were included in the CDC Fusarium keratitis outbreak investigations, these species have not yet been observed to occur in epidemics or cause outbreaks. Concerning geography, 51 clinical isolates were recovered from the United States, and this revealed that phylogenetically diverse human opportunists are well represented in North America.40 In our study, three clinical *F. equiseti* strains originated from Mexico, and this might suggest that species of this complex are common in this region. The virulence of members of the FIESC has been ascribed to their production of type A and B trichothecene mycotoxins.39

*F. dimerum* and *F. delphinoideos* belong to the *F. dimerum* species complex, and both were isolated from superficial and disseminated infections.15 In our data set, a supported clade of FDSC matching with the AFLP data mainly contained strains from India, and this might suggest a regional prevalence. *F. chlamydosporum* was reported in disseminated infections in patients with aplastic anemia and lymphocytic lymphoma from the United States.41,42 CBS 111770 (*F. concolor*) is the only clinical strain in the *F. concolor* species complex, and it was reported in a keratitis case from Spain.43

By comparing AFLP and MLST data, *F. falkumori* and *F. keratoplasticum* appear to be widely distributed, at least in Mexico, North America, Europe and India, with dominancy in superficial infections, including keratitis and onychomycosis. *F. petrophilum* is the second most diverse species and is also frequently involved in disseminated infections. *F. solani sensu stricto*, ‘5’, which was recently described as *Fusiporium (Fusarium) solani* (FSSC5),7 contains strains such as CBS 135559, CBS 135564 and CBS 135565, which originate from Mexico, and shows significant occurrence in keratitis cases. This species was also recently reported in Asia (India and Qatar).13,16 Given the large distances of identical strains occurring in many different countries, airborne distribution seems likely. However, the presence of *F. incarnatum, F. equiseti* and *F. chlamydosporum* in clinical samples...
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from various infections in North America remains puzzling but can perhaps be explained by sampling effects.

As previously noted, the F. fujikuroi complex contains the highest number of species. In our study, 15 supported clades were recognized in all of the molecular analyses (Figures 1 and 2). Nearly all of the clades have various geographic distributions. Within the F. fujikuroi species complex, F. proliferatum and F. verticillioides were the dominant clinically relevant species, having a global distribution and dominating in disseminated infections. F. sacchari is the second most prevalent species and was often isolated from keratitis restricted to India. Although F. nygmaei and F. napiforme are the most multidrug-resistant species within the F. fujikuroi complex, their presence in human infections is rare. F. acutatum was reported from nail infections in four cases in Qatar, showed a low degree of variability and has been suggested to be clonal. These results emphasize that F. acutatum is an emerging human opportunist, which thus far was only detected in Asia. Sequence analysis of RPB2 and TEF1, and AFLP showed that the strains CBS 119850, CBS 483.94 and CBS 454.97 were nested within the F. fujikuroi complex and close to F. nygmaei and F. anidyi, forming a well-supported monophyletic branch suggestive of a novel species.

Deep fusariosis is rare in healthy individuals; a single brain infection has been reported. Local infections may occur after a direct inoculation or tissue breakthrough by trauma or the entrance of foreign bodies. The treatment of superficial infections is usually successful and requires surgery, the removal of the foreign body and antifungal therapy. The most important risk factors for severe fusariosis are prolonged neutropenia and T-cell immunodeficiency in patients suffering from a hematological malignancy. Fusarium infections in the majority of these cases were due to neutropenia. Furthermore, in solid organ transplant recipients and cancer patients with neutropenia, infections due to Fusarium spp. increased and led to disseminated infection. Patients develop painful skin lesions, which vary from papules to nodules with or without central necrosis. In the majority of disseminated infections, secondary skin lesions led to a diagnosis in >50% of the patients and preceded fungemia by ~5 days. In contrast to aspergillosis, fusariosis frequently shows positive blood cultures because Fusarium conidia are hydrophilic and allow dissemination. Comparing fusariosis with mucormycosis, solid tumors and diabetes do not seem to be important risk factors. Only 17 (4.8%) cases were found in patients with solid tumors, and seven infections were reported in patients with diabetes mellitus. No underlying conditions were observed in 20 (5%) of the cases.

Fusarium treatment depends on the site of infection. Surgery with antifungals was used in 80 cases (20.6%). Disseminated fusariosis in immunocompromised patients is usually treated with amphotericin B and voriconazole as the first-line therapy, which is suggested by recent guidelines. In our literature review, most antifungal therapy was amphotericin B deoxycholate, followed by liposomal amphotericin B and voriconazole. The most commonly used combination is amphotericin B/voriconazole followed by amphotericin B/S-flucytosine. Triple combinations were used in 14 cases with different antifungals.

The major findings of the present study include the following: (i) human-associated fusaria were nested within seven species complexes (that is, F. chlamydosporum, F. concolor, F. dimerum, F. fujikuroi, F. incarnatum-equisetii, F. oxysporum and F. solani), (ii) the three most common species presented in both the clinical and environmental groups are F. falciforme and F. keratothrix (members of F. solani species complex) followed by F. oxysporum (FOSC), (iii) most of the reported Fusarium species in this study were shared among the patients and the environment, and this might be due to the colonization of some patients with Fusarium isolates from the environment; hence, there is genetic similarity between the clinical and environmental isolates of the same Fusarium species, and (iv) the species distributions show some evidence of geographical clustering among some of the species studied, although the present study is limited by an over-representation of isolates from Mexico and India.

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