



Utility of a proposed CSP typing nomenclature for Australian *Aspergillus fumigatus* isolates: Identification of additional CSP types and suggested modifications

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ABSTRACT

A recently developed CSP typing scheme and proposed nomenclature was applied to a collection of 164 clinical and environmental *Aspergillus fumigatus* isolates from Melbourne, Australia. Fifteen CSP variants were observed overall, including three that were not reported in the original nomenclature that described 19 CSP variants, raising the possibility of phylogeographic differences between the Australian and the previously studied European and North American *A. fumigatus* populations. However, those CSP variants that were common between this and the previous studies appeared to have a broadly similar prevalence. The presence of an additional CCT codon in the 3' flanking region of some CSP variants was also observed in homologous *Neosartorya fischeri* sequence, suggesting that the absence of this codon in other isolates is due to codon deletion, rather than its presence representing a duplication. We recommend a number of modifications to the proposed CSP type nomenclature to accommodate these new findings.

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1. Introduction

Aspergillus fumigatus is an opportunistic, filamentous fungus that is ubiquitous in the environment, and is associated with major morbidity and mortality in immunocompromised patients. Numerous molecular epidemiology studies of this pathogen have been conducted in Europe and North America (Debeauvais et al., 1997; Chazalet et al., 1998; Cimon et al., 2001; Bertout et al., 2001; Warris et al., 2003; Bain et al., 2007; Vanhee et al., 2008; de Valk et al., 2009), to examine the mechanisms of nosocomial outbreaks and to gain insight into the extent of genetic diversity, relationships between clinical and environmental isolates, and patterns of colonization and invasive infection in patients. However, little is known about these aspects of the *A. fumigatus* population in Australia, or the similarities and differences between Australian isolates and those in other areas of the world. The use of a wide variety of non-standardized techniques in molecular epidemiological studies of *A. fumigatus* has to date made data comparisons near impossible.

CSP typing is a recently developed typing strategy that overcomes many of the problems concerning data comparison. This method involves DNA sequence typing of a repetitive region of the *A. fumigatus* AFUA_3G08990 gene, designated the CSP locus (Balajee et al., 2007).

The CSP locus comprises a series of tandemly repeated 12-mer motifs, among which a number of repeat types have been identified. Variation between isolates arises due to insertion or deletion of repeat types, resulting in differences in the number and organization of repeats. Additional sequence variation resulting in strain discrimination can be observed in the flanking regions of the tandem repeats. The *in vitro* stability of the CSP sequence has been demonstrated, and both the intra- and inter-laboratory reproducibility have been established (Balajee et al., 2007; Hurst et al., 2009). The discriminatory power of CSP typing is intermediate to MLST and STRAf microsatellite typing (Bain et al., 2007; de Valk et al., 2005), and as such has been recommended as a useful first-line approach to typing *A. fumigatus* isolates and determining relationships at the sub-population level (Klaassen et al., 2009).

In a recently published study, 18 CSP types were described, based upon CSP locus variation from 9 repeat types. In addition, CSP sub-types were discerned from variation in the flanking sequence, bringing the total number of recognised CSP sequence variants to 19 (Klaassen et al., 2009). Eighteen of these CSP variants were recognised among 209 *A. fumigatus* isolates from clinical specimens at 14 centres in The Netherlands and one more was recognised among 55 epidemiologically linked isolates from North America (Balajee et al., 2007; Klaassen et al., 2009). Based upon these findings, a standardized nomenclature for recognition of these CSP sequence variants was proposed (Klaassen et al., 2009).

In this study we applied the CSP typing strategy and the proposed nomenclature, for the first time to Australian clinical and environmental *A. fumigatus* isolates, to determine the extent of genetic variation, and

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for comparison to previously investigated isolates from North America and The Netherlands (Balajee et al., 2007; Klaassen et al., 2009).

2. Materials and methods

2.1. Isolates

A total of 164 isolates were investigated in this study; all were identified as *A. fumigatus* by macroscopic and microscopic characteristics, as well as by sequencing of the ITS1–5.8S–ITS2 rDNA region (White et al., 1990). There were 136 clinical isolates included: 125 were collected from 41 patients at The Alfred Hospital, Melbourne between November 2007 and February 2009 as part of a prospective surveillance study, and an additional 11 isolates were obtained from 10 patients at The Alfred Hospital and the Peter MacCallum Cancer Centre, Melbourne in 2006 as part of investigations into potential nosocomial outbreaks. Between 1 and 14 isolates were included for each patient. In addition, 28 environmental isolates were included in the study: 21 isolates were cultured from nine soil samples from the grounds of The Alfred Hospital between August 2008 and February 2009, and 7 isolates were obtained from air and surface samplings within the Peter MacCallum Cancer Centre in 2006.

2.2. DNA isolation

Genomic DNA was isolated from conidia harvested from cultures grown at 30 °C for 3–4 days. DNA extraction was performed as previously described (Jin et al., 2004), and was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Barrington, IL, USA).

2.3. CSP typing

The CSP locus was amplified in a reaction volume of 25 µl, containing 25 ng of genomic DNA (10 ng/µl), 1 µM each of the primers 5'-TTG GGT GGC ATT GTG CCA A-3' and 5'-GGA GGA ACA GTG CTG TTG GTG A-3' (Invitrogen, Mt. Waverly, VIC, Australia), 0.2 µM dNTPs, 1× buffer II (Invitrogen), 0.5 µl AccuPrime Taq DNA Polymerase (Invitrogen). The PCR cycling comprised an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s, and 68 °C for 1 min, followed by a final extension at 68 °C for 10 min, and cooling to 4 °C. PCR products were purified using the MinElute Gel Extraction kit (Qiagen, Doncaster, VIC, Australia). Sequencing reactions were performed using a modification of the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Scoresby, VIC, Australia). Forward and reverse sequence reactions were performed in a volume of 20 µl, containing 20 ng of purified PCR product, 5 pmol of either the forward or reverse primer used for PCR amplification, 0.875× reaction buffer, and 1 µl of Big Dye premix. Sequence cycling conditions were according to the manufacturer's instructions. Sequencing reaction products were purified by ethanol precipitation before being analyzed on the 48-capillary 3730 DNA sequencing platform (Applied Biosystems).

2.4. DNA sequence analysis

Sequences were manually edited and aligned using the BioEdit v7.0.9 software. Repeat types and CSP sequence types were assigned to isolates as defined in the proposed nomenclature (Klaassen et al., 2009). For the purposes of outgroup comparison, homologous sequence from the closely related species, *Neosartorya fischeri* was obtained from the NRRL 181 genome assembly (<http://www.tigr.org/db/e2k1/nfa1/>; NCBI accession XM_001263541).

2.5. STRAf typing

The highly discriminatory STRAf microsatellite typing method was used to distinguish between different strains of *A. fumigatus* isolated

Table 1

Summary of the CSP 12-mer repeat type sequences observed to date.

Repeat type	Repeat sequence
r01	ACT TCT GTC CCG
r02	ACT TCT GTC CCA
r03	ACT CAA AAC GCG
r04	ACT TCA ATC CCG
r05	ACT TTT GTC CCG
r06	ACT TCA GTC CCG
r07	ACT ACT ATT GTG
r08 ^a	ACT TTT CTC CCG
r09	ACT TCT GTT CCG
r10 ^b	ACT TCA ATC CCA
nf1 ^c	ACT CAG AAC GCG

^a Repeat type r08 not observed among the Australian isolates in this study.

^b Repeat type r10 not previously reported.

^c Repeat type nf1 not previously reported and was observed only in the *N. fischeri* outgroup sequence. Included for comparative purposes only, and not considered to be part of the *A. fumigatus* CSP type nomenclature.

from the same patient or primary culture plate. The previously described protocol was followed (de Valk et al., 2005), with the exception that the microsatellites were analyzed using the 96-capillary 3700 DNA Analyzer (Applied Biosystems), and primers were labelled with fluorescent dyes appropriate for this platform. Simpson's index of diversity (Simpson, 1949) was used to calculate the observed genetic diversity among isolates, from which the discriminatory power of the typing methods was also inferred.

3. Results

A total of 164 Australian clinical and environmental *A. fumigatus* isolates were analyzed by CSP typing in this study and 100% typeability was achieved.

As recommended in the proposed CSP type nomenclature, we examined the nucleotide sequence of each tandem repeat. Table 1 lists the repeat types observed among the Australian isolates investigated in this study. We identified 9 distinct repeat types, 8 of which were identified among the 209 Dutch isolates previously described (Klaassen et al., 2009). The additional repeat type, which we have designated r10 (see Table 1), has not previously been reported. The previously described r08 repeat type was not observed among isolates in this study.

By inspection of the DNA sequences within the tandem repeat region as well as the –45 bp and +9 bp flanking regions, we identified a total of 15 CSP variants, which are summarized along with all previously recognised CSP variants in Table 2. Three CSP variants (designated here as t19, t20, and t06B) observed among the isolates in this study were not previously described among isolates from The Netherlands or North America (Balajee et al., 2007; Klaassen et al., 2009). CSP type t19 was observed among nine isolates from four patients and one soil sample, and contains 16 repeat units, including the novel repeat type r10. CSP type t20 was observed for one clinical isolate, and has the shortest tandem repeat sequence observed to date, containing only four 12-mer repeat units. CSP sub-type t06B, which was observed for two isolates from one patient, has the same tandem repeat sequence as t06A (previously designated t06; Klaassen et al., 2009), but differs in its flanking sequence; t06B has CTC rather than GTC at codon –14, CCG rather than CCA at codon +1, as well as what has been described as a duplication of codon +2 (see Table 2).

Comparison of *A. fumigatus* CSP sequences to homologous sequence from its sibling species, *N. fischeri*, revealed a comparable pattern of tandem repeat units with a high degree of sequence identity to *A. fumigatus*. The *N. fischeri* sequence contained four 12-mer repeat units, one of which was not observed in any of the *A. fumigatus* isolates, and was designated 'nf1' for the purposes of this

Table 2

Summary of tandem repeats and flanking sequence for the 22 CSP types identified among *A. fumigatus* isolates to date.

CSP type	Codon			Tandem repeat succession	Codon		
	–15	–14	–1		+1	+2	+3
t01	GTG	GTC	CCG	01-01-01-01-01-05-03-01-06-03-07	CCA	CCT	
t02	GTG	GTC	CCG	01-01-02-03-04-05-03-01-06-03-07	CCA	CCT	
t03	GTG	GTC	CCG	01-02-03-04-06-03-07	CCA	CCT	
t04A	GTG	GTC	CCG	01-02-03-04-05-03-01-06-03-07	CCA	CCT	
t04B ^c	GTG	GTC	CCA	01-02-03-04-05-03-01-06-03-07	CCA	CCT	
t05	GTG	GTC	CCG	01-01-01-03-01-06-03-07	CCA	CCT	
t06A ^a	GTG	GTC	CCG	01-01-01-02-03-04-05-03-01-06-03-07	CCA	CCT	
t06B ^b	GTG	<u>C</u> T <u>C</u>	CCG	01-01-01-02-03-04-05-03-01-06-03-07	<u>CCG</u>	CCT	<u>CCT</u>
t07 ^c	GTG	<u>C</u> T <u>C</u>	CCG	01-02-03-04-05-03-04-05-03-01-06-03-07	<u>CCG</u>	CCT	<u>CCT</u>
t08 ^c	GTG	<u>C</u> T <u>C</u>	CCG	01-01-01-02-03-04-05-03-04-05-03-01-06-03-07	<u>CCG</u>	CCT	<u>CCT</u>
t09	GTG	GTC	CCG	01-01-01-01-01-05-03-01-06-03-07	CCA	CCT	
t10	GTG	GTC	CCG	01-01-01-05-03-01-06-03-07	CCA	CCT	
t11 ^c	GCG	<u>C</u> T <u>C</u>	CCG	01-01-08-03-01-06-03-07	CCA	CCT	
t12 ^c	<u>G</u> T <u>G</u>	<u>G</u> T <u>C</u>	CCG	01-01-01-01-01-02-03-04-05-03-01-06-03-07	CCA	CCT	
t13	GTG	<u>C</u> T <u>C</u>	CCG	01-01-02-03-04-05-03-04-05-03-01-06-03-07	<u>CCG</u>	CCT	<u>CCT</u>
t14	GTG	GTC	CCG	01-01-01-01-02-03-04-05-03-01-06-03-07	CCA	CCT	
t15 ^c	GTG	<u>C</u> T <u>C</u>	CCG	01-01-01-01-02-03-04-05-03-04-05-03-01-06-03-07	<u>CCG</u>	CCT	<u>CCT</u>
t16 ^c	GTG	GTC	CCG	01-05-03-01-06-03-07	CCA	CCT	
t17	GTG	GTC	CCG	01-01-02-03-04-05-03-09-06-03-07	CCA	CCT	
t18	GTG	GTC	CCG	01-01-05-03-01-06-03-07	CCA	CCT	
t19 ^b	GTG	<u>C</u> T <u>C</u>	CCG	01-01-02-03-10-03-04-05-03-04-05-03-01-06-03-07	<u>CCG</u>	CCT	<u>CCT</u>
t20 ^b	GTG	GTC	CCG	01-02-03-07	CCA	CCT	
<i>N. fischeri</i> ^d	GTG	GTC	CCG	01-01-nf1-07	<u>CCG</u>	CCT	<u>CCT</u>

^a CSP type t06A was previously designated as t06 (Klaassen et al., 2009).

^b CSP types not reported among isolates in previous studies.

^c CSP types not observed among the Australian isolates in this study.

^d *N. fischeri* CSP sequence is included for comparative purposes only, and is not considered to be part of the *A. fumigatus* CSP type nomenclature. Observed sequence variation in the flanking regions of the tandem repeats is indicated by an underline.

study (Table 1). The –45 bp and +9 bp flanking regions of the *N. fischeri* CSP locus were highly similar to those of *A. fumigatus* (Table 2), and like six of the *A. fumigatus* CSP variants recognised to date, *N. fischeri* possessed an additional CCT codon in the +9 bp flanking region.

A total of 105 genotypes were distinguished using STRAf typing among the 164 *A. fumigatus* isolates included in the study. Eighty-one of these were unique to a single isolate, while 24 were common to two or more isolates. Considering the clinical isolates, where a total of 81 genotypes were observed, 71 were unique to a single patient, while 10 were shared by up to four patients each. All isolates with the same STRAf genotype possessed the same CSP type, indicating concordance between these techniques.

The prevalence of each CSP type among the Australian isolates was calculated, and is summarized in Table 3; identical strains as determined by STRAf typing, from the same patient or from the same environmental primary culture plate were removed from this analysis to reduce clone bias. The most commonly observed CSP variants were t04A, t03, and t01, while the least commonly observed were t06B, t14, t17, and t20. The previously described CSP types t07, t08, t11, t12, t15, t16 (Klaassen et al., 2009), were not observed among the isolates in this study. We observed no significant differences in the prevalence of CSP variants between clinical and environmental isolates. The observed genetic diversity (and hence, the discriminatory power of the techniques) among the Australian isolates was calculated at 0.8128 using CSP types and 0.9968 using STRAf genotypes.

4. Discussion

This study represents the first molecular typing investigation of *A. fumigatus* isolates from Australia, and given that CSP typing has so far been used to describe only 264 isolates from North America and The Netherlands, adds significantly to the data that has previously been reported (Balajee et al., 2007; Klaassen et al., 2009). The CSP typing technique was successfully applied to all isolates in this study, and has confirmed the utility of this technique, the usefulness of the CSP typing nomenclature for inter-laboratory comparisons, and its concordance with STRAf genotypes (Balajee et al., 2007; Balajee et al., 2008; Klaassen et al., 2009). Importantly, we have identified three CSP variants among the Australian isolates that were not observed among the North American or Dutch isolates.

CSP typing is a highly reproducible and stable technique, and its discriminatory power is intermediate to MLST and STRAf typing, making it ideal for use as a first-line approach for typing *A. fumigatus* isolates and for determining relationships at the sub-population level (Balajee et al., 2007; Bain et al., 2007; Hurst et al., 2009; Klaassen et al., 2009). However, until recently the various CSP types were poorly defined in terms of the specific region of the locus being examined, the nature of the 12-mer repeat types, and the overall range and designations of sequence variants. The proposal for a standardized nomenclature for the CSP typing system based upon the highly successful *spa* typing system in use for *Staphylococcus aureus* strains (Klaassen et al., 2009; Harmsen et al., 2003), will greatly simplify the recognition and communication of CSP type data. However, in light of the new findings from our study, we recommend a number of modifications to the proposed CSP typing nomenclature.

In this study we observed one repeat type (t10) and three CSP variants (t19, t20, and t06B) that have not previously been reported, increasing the total number of distinct CSP variants observed to date to 22. This includes the previous t06 type, which we suggest now be recognised as t06A to distinguish it from the t06B sub-type. As part of maintaining a standardized nomenclature for CSP types and of facilitating an understanding of the genetic diversity of *A. fumigatus*, it is important that these additional CSP types be recognised, as well as others that are likely to be identified in the future, as the application of this technique becomes more widespread. A publicly accessible online database, such as that which is in place for the *S. aureus spa* typing system (<http://www.seqnet.org/spaserver.shtml>), would facilitate not only sequence comparisons and determining the CSP types of isolates, but also the rapid reporting of new sequence variants as they are discovered.

Table 3

Prevalence of CSP types observed among the Australian clinical and environmental isolates.

CSP type	Prevalence (%) ^a		
	Clin. (n = 95)	Env. (n = 27)	Overall (n = 122)
t01	22.1	18.5	21.3
t02	7.4	7.4	7.4
t03	26.3	14.8	23.8
t04A	25.3	40.7	28.7
t05	2.1	0	1.6
t06A	3.2	0	2.5
t06B	1.1	0	0.8
t09	1.1	7.4	2.5
t10	2.1	0	1.6
t13	0	7.4	1.6
t14	1.1	0	0.8
t17	1.1	0	0.8
t18	2.1	0	1.6
t19	4.2	3.7	4.1
t20	1.1	0	0.8

Abbreviations: Clin – Clinical isolates, Env – Environmental isolates.

^a Duplicate strains from the same patient or from an environmental primary culture plate, as identified by STRAf typing, were removed from this analysis.

Klaassen et al. (2009) reported what was referred to as a duplication of the CCT codon within the +9 bp flanking region in some CSP types (t07, t08, t13, and t15). This codon was also observed among isolates in this study (t06B and t19), as well as in the *N. fischeri* outgroup sequence. Assuming that *N. fischeri* and *A. fumigatus* have not both independently undergone codon duplications at this position, this suggests that the presence of the additional codon actually represents the ancestral state, and a codon deletion is the more parsimonious explanation for its absence in other isolates. Therefore, we suggest referring to this as the +3 codon in the CSP nomenclature, rather than as a duplication of the +2 codon as was previously recommended.

To date the observed CSP variants have been designated either as 'types' or 'sub-types', depending on whether sequence variation is observed within the tandem repeat sequence or within the flanking regions. However, this implies a hierarchical importance in the location of the sequence variation, which is not relevant to the fact that any observed sequence variation represents a distinction between strains. Furthermore, CSP sub-types as defined (Klaassen et al., 2009), are not necessarily phylogenetically closely related and referring to them as such could be misleading. For example, while t06A and t06B possess the same tandem repeat sequence, t06A is distinguished from t06B by the deletion of the +3 CCT codon as well as by having different codons at the -14 and +1 positions. This implies significant genetic differences between the t06A and t06B sub-types that could potentially go unrecognised by referring to them as sub-types. Since the ultimate aim of the CSP nomenclature is to simplify and facilitate recognition and communication of *A. fumigatus* genetic diversity, then treating all CSP variants as distinct, equally weighted entities, would perhaps enable the most effective implementation of the nomenclature. While there is fundamentally no problem in assigning a suffix to CSP variants to indicate that the observed variation is located within the flanking sequence, we recommend referring to all CSP variants as 'CSP types', without further classifying them as a type or sub-type.

The most prevalent CSP types observed in this study were also the most prevalent in the Dutch and North American studies (Klaassen et al., 2009). Those CSP types that were not observed among the isolates in this study had a relatively low prevalence in the Dutch study (Klaassen et al., 2009), and their absence in this study may be a reflection of sample size. We have so far examined only isolates from Melbourne, a city with a temperate climate and a population of four million, and have not yet sampled widely across Australia. Similarly, the three CSP types that have so far been observed only among Australian isolates may be due to sampling artefact. Alternatively, the differences in the range of CSP types observed within these populations may represent phylogeographic distinctions between the European, North American and Australian *A. fumigatus* populations. Expansion of our prospective surveillance study to other centres in Australia and New Zealand is in progress, and this will enable us to ascertain a more accurate insight into the range of CSP types and their prevalence in this region. Additionally, it will be important to perform a collaborative global study of *A. fumigatus* isolates to establish a comprehensive and representative set of CSP type definitions. This will also allow us to confirm or refute phylogeographic differences between

the Australian *A. fumigatus* population and those of Europe and North America, as well as to examine population structure in different regions. Given the amenability of CSP typing to inter-laboratory data comparisons, such a study would be relatively easy to conduct.

In summary, while we have confirmed and validated the utility of CSP typing when applied to a different population from that in which it was derived, our findings lead us to recommend a number of modifications to the proposed CSP typing nomenclature that will simplify its application.

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