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Discovery of a sexual cycle in Aspergillus clavatus

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Abstract

Background and objective: Aspergillus clavatus is an opportunistic human pathogen causing invasive aspergillosis. It is an economically important species because it can grow on rotting fruit (apples) and stored food products and able to produce a variety of mycotoxins. It has only been known to reproduce mitotically. This study aimed to discover the sexual reproduction in A. clavatus.

Methods: Identifying mating-type (MAT1-1 or MAT1-2) using PCR of 20 worldwide clinical and environmental isolates, crossing isolates on oatmeal agar in darkness with plate sealing, Recombination in ascospore offspring was demonstrated using molecular markers.

Results: There were similar ratios of the two mating types (45% MAT1-1 n = 9, 55% MAT1 -2 n **=** 11). A. clavatus possesses a functional sexual cycle with mature cleistothecia fawn to brown/yellow in color, containing heat-resistant ascospores, produced after four weeks incubation at 25˚C and 28˚C on Nescofilm- sealed oatmeal agar plates. The cleistothecia contain hyaline ascospores that have two equatorial ridges. Recombination, leading to increased genotypic variation demonstrated in the ascospore offspring using molecular markers.

Conclusion: The ability of A. clavatus to undergo sexual reproduction is highly significant in understanding the biology and evolution of the species. The presence of a sexual cycle provides an invaluable tool for classical genetic analysis and will facilitate research into the genetic basis of pathogenicity and fungicide resistance.

Keywords: Cleistothecium; Ascospore; Aspergillus clavatus.

Introduction

The genus Aspergillus includes both sexual and asexual species, with both homothallic and heterothallic species evident, meaning that the genus is ideal to study the evolution and development of sexual breeding systems. Until recently the majority of Aspergillus species, up to 20% of fungal species, especially those related to the phylum Ascomycota, had no known mode of sexual reproduction, one such asexual species of medical and economic importance Aspergillus clavatus which is an opportunistic human pathogen causing invasive aspergillosis. A. clavatus is of particular interest because it is both of medical and economic importance. The species has been implicated in human mycoses, as a causal agent of opportunistic infections in immunocompromised patients.[1] It also causes extrinsic allergic alveolitis known as farmer's or malter's lung,[2] since the fungi survive the malting process, and some of the mycotoxins retain functionality even after the kilning process used in malting.[2,3] The reason why A. clavatus is not an invasive pathogen like A. fumigatus, and rather causes extrinsic allergic alveolitis, seems to be because A. clavatus grows more slowly at 37°C than A. fumigatus and the species also has a larger spore size, which may prevent deep penetration of the lung.[4] A. clavatus also causes

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endocarditis,[5] and other mycotoxicoses-related disease observed in sheep and cattle fed with infected grain[6,7] and various toxic syndromes including neurological disorders.[8,9] Its spores are carcinogenic in mouse lung.[10] A. clavatus is also an economically important species because it can grow on rotting fruit (especially apples) and stored food products (mainly cereal grains) with high moisture content e.g. inadequately stored rice, corn and millet[2,3] and their malts (germinated seeds) including barley, maize and sorghum, as well as other stored feedstuffs, including legume seeds. The species also occurs frequently in pigeon droppings.[11,12] The ability of A. clavatus to grow in highly alkaline conditions contributes to its ubiquitous occurrence in dung and certain soils worldwide. For decades, A. clavatus has been considered to be asexual species. However, there are various indicators that the species might have a cryptic sexual state. Firstly, the section Clavati forms a basal clade to species in the section Fumigati and the genus Neosartorya which includes both homothallic and heterothallic sexual species.[13] In particular, the section Clavati includes a known homothallic sexual species, classified in the teleomorph genus Neocarpenteles.[1,14] This species, Neocarpenteles acanthosporus (anamorph: A. acanthosporus), is the only known teleomorph in this section but indicates the presence of extant sexuality in this group. The species produce hard and sclerotioid fawn nonostiolate, unilocular stromata in which asci are gradually produced outward from the center during sexual reproduction. These take 3-4 weeks to mature and contain hyaline ascospores that have two equatorial ridges.[14] The second indicator of latent sexuality in A. clavatus has come from genome studies. The genome of A. clavatus has been sequenced and a genome size of 27.9 Mb determined, which is the smallest seen to date among the aspergilli, comprising a putative 9,125 genes.[15] BLAST analysis revealed the presence of a suite of apparently functional 'sex-related' genes in the genome including a MAT idiomorph.[16] Further analysis of a series of natural isolates then revealed the presence of isolates with either MAT1-1 or MAT1-2 idiomorphs, consistent with heterothallic sexuality.[16] Given the "sexual revolution" observed in other asexual aspergilla.[17] It, therefore, seems possible that A. clavatus might have a hidden, cryptic sexual cycle which would provide a valuable genetic tool for the study of its pathogenicity and mycotoxin production. This study aimed to discover the sexual reproduction in A. clavatus.

Methods

Strains and DNA extraction

Twenty isolates of A. clavatus were used in this study which was obtained from stocks held in the BDUN collection at the University of Nottingham. Strains were maintained on Aspergillus complete medium (ACM)[18] at 28°C. DNA was extracted using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

Mating-type assays and sexual crosses

To study the potential for sexual reproduction in A. clavatus it was first necessary to elucidate the mating types of strains so that directed crosses could be set up. Therefore, all strains were screened for the presence of either MAT1-1 or MAT1-2 mating-type genes following a standard protocol (Table 1).[16]

Table 1: Primers used in the multiplex mating-type PCR assay for A. clavatus.

Primers	Sequence 5' to 3'
AclM1F	CAGTTGTTCGTAGCAGACGGGG
AclM1R	CGGTGGAGTATGCTTTGGCGAGG
AclM2F	ATCAAGGCTCTTCGTGTCATGC
AclM2R	ATGCTTCTTCTTCATATCTTCTGCC

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All PCR reactions were performed using 25 µl reaction volumes containing 2.5 µl 10X PCR Buffer (containing 20mM MgCl2), 0.2 µl (25mM each) dNTPs, 2.5 µl (10µM) of respective MAT forward primer, 2.5 µl (10µM) of respective MAT reverse primer, 0.2 µl FastStartTaq Polymerase (Roche, UK), ~50ng genomic DNA, and ultrapure water to a final volume of 25 µl. The amplification of fragments was performed on a Techne Genius thermal cycler, using the following cycling parameters: an initial denaturation step at 94°C for 5 min; 35 cycles consisting of 1 min at 94°C, 1 min at 60°C for MAT1-1 and 55°C for MAT1-2 and 1 min at 72°C; followed by a final extension step at 72°C for 5 min, all steps were performed at a ramp rate of 70°C/min. Resultant PCR products were resolved on 1.5% agarose gels and visualized by ethidium bromide staining For analysis of the putative MAT1-1-1 gene primer pair AclM1F and AclM1R was used, which was predicted to yield a 244bp product; the putative MAT1-2-1 gene was analysed using the primer pair AclM2F and AclM2R, which was predicted to yield a 388bp fragment. Crosses were set up in 9 cm petri dish between six MAT1-1 strains and three MAT1-2 strains which were inoculated in all possible pairwise combinations (n = 18).[19] All crosses were set up on oatmeal agar; [20] pinhead, Odlums, Ireland in triplicate, sealed with Nescofilm® and incubated at 25°C, 28°C, 30°C in the dark. The crosses were examined periodically for the presence of cleistothecia for up to five months, using a Nikon-SMZ-2B dissection microscope.

Preparation of single ascospore cultures

Mature cleistothecia from the cross 65-14 x 65-16 were removed, and ascospores suspension were prepared. The suspension of conidia and ascospores of A. clavatus was first heat shocked at 80°C for 30 minutes to inactivate any contaminating conidia as previously described for both A. fumigatus and A. lentulus. However, this was found to result in killing of both conidia and ascospores, unlike in A. fumigatus and A. lentulus. Therefore, a range of more gentle treatments was used involving heat shock at lower temperatures (68°C, 69°C, 70°C, 72°C, 75°C, and 80°C), and for a shorter time (10 min). Finally, an alternative heat shock was used whereby cleistothecia were cleaned by rolling on tap water agar to remove adhering conidia and were then added to the 500 µl of 0.05% (v/v) tween 80, which was then heat-treated at 69°C for 10 min before squashing. The cleistothecia were separated by centrifugation, the supernatant discarded and then 50 µl of 0.05% tween 80 was added and cleistothecia ruptured by squashing with a needle tip. The solution was then brought up to 500 µl by the addition of 0.05% tween 80 and vortex-mixed for 1 min to release the ascospores. One hundred µl of a $5x10^5$ ascospore ml^{-1} heat treated suspension was spread inoculated on ACM plates. Triplicates were prepared and incubated at 37°C for 14 h. Single spore cultures were established on ACM by transferring individual germinating ascospore with a LaRu lens cutter attached to a (Nikon-OPTIPHOT) microscope.

Evidence of recombination

The segregation of five genetic markers (four RAPD bands and the mating –type genotype) in the ascospore progeny was examined using RAPD-PCR fingerprinting as describe (RAPD-PCR was performed according to Murtagh,[21] using DyNAzyme II DNA Polymerase (Finnzymes, Finland). Each 25 µl reaction contained ~1 ng genomic DNA, 2.5 µl 10X PCR Buffer, (containing 1.5mM $MgCl_2$), 0.15 µl dNTPs (25 mM each), 0.1 µl of a single 10-base-pair RAPD primer (50µM) (see Table 2), 0.375 µl of DNA Polymerase (2U/µl), and 20.875 µl ultra pure water. Blank control tubes containing all reagents but substituting the template DNA for ultra pure water were also included in each run. The amplification of fragments was performed on a Techno Genius thermal cycler using the conditions described in[21]

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45 cycles of 93 °C for 30 s (ramp rate 30 ° C min^{-1}), 37 °C for 40 s (ramp rate 30 °C min^{-1}) and 72°C for 1 min 20 s (20 ºC min^{-1}) before a final elongation stage of 72 °C for 5 min and storage at 4°C. Amplification products (12 µl of the total reaction) were then analyzed by electrophoresis in 1.5 % (w/v) agarose gels containing TBE buffer for approximately 2 hours at 100 V and sized using a 1 kb DNA ladder. Gels were stained with ethidium bromide (1 µl/ml) after which bands were visualized via UV light and photographed using a BioRad Chemidoc XRS (Bio□Rad Laboratories, UK). An initial screen of sixteen primers revealed four (OPC20, 0PT18, UBC90, OPQ6) that yielded polymorphisms suitable for genotyping (Table 2) [19].

Statistical analysis: The hypothesis of a 1:1 ratio of mating types in the worldwide sample population and ascospore progeny was tested using χ^2 and contingency χ^2 tests (P <0.05). Where expected frequencies were less than five, Fisher's exact test was used.[22]

Scanning electron microscopy

Cleistothecia were collected from six-month -old crosses of 65-14 x 65-16 that had been incubated at 25 °C and examined by scanning electron microscopy, cleistothecia crushed in a drop of sterile water were transferred onto 0.2 µm filter discs (Whatman) and fixed in 2% (w/v) osmium tetroxide (Sigma) for 2 hours at room temperature until all the osmium tetroxide had evaporated. The fixed samples were then mounted onto aluminum stubs using double-sided conductive carbon tabs, dried at 37 °C overnight and sputter-coated with gold. SEM micrographs were taken using a JSM -840 Jeol scanning electron microscope and processed with (Adobe Photoshop CS5.1 software).

Results

The MAT PCR diagnostic[16] using primer pairs AclM1F with AclM1R, or AclM2F with AclM2R successfully amplified putative MAT gene fragments from all 20 worldwide isolates of A. clavatus. Amplicons of the predicted 244bp size for MAT1-1 genotypes and 388bp for the MAT1-2 genotypes were produced in different isolates (Figure 1).

Table 2: Primers used for recombination analysis of A. clavatus.

Primers	Sequence 5' to 3'
UBC90	GGGGGTTAGG
OPC-20	ACTTCGCCAC
OPT-18	GATGCCAGAC
OPQ-06	GAGCGCCTTG

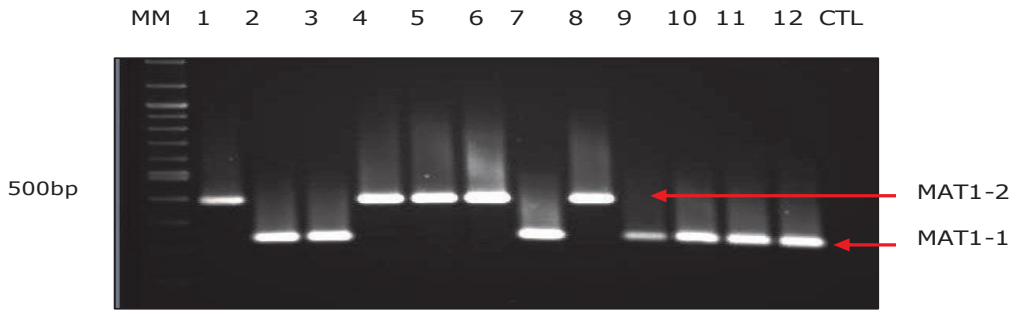

Figure 1: The MAT PCR diagnosis, 1.5% agarose gel showing representative results of PCR, amplification of A. clavatus to determine whether of MAT1-1 or MAT 1-2 genotype. Molecular weight marker (MM); lanes (1→12) are amplicons of A. clavatus, water control (CTL). Arrows point to mating-type specific 388bp (MAT1-2) or 244bp (MAT1-1) bands, bp-base pair.

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The worldwide collection of isolates was then screened to determine the ratio of complementary MAT1-1 and MAT1-2 genotypes. Amplicons were generated for all isolates and the overall mating-type distribution did not deviate significantly from a 1:1 ratio [(45 % MAT1-1 n=9, 55 % MAT1-2 n=11); X^2 = 0.80; n = 20; *P* = 0.654]. When isolates were grouped according to geographic origin, there was also no significant difference in the MAT distribution. Based on the equal distribution of mating types, it can be assumed that these populations previously or currently are propagating sexually in their original habitats.

Sexual development and reproductive structures

Nine A. clavatus strains (6 MAT1-1 and 3 MAT1-2) from different geographic origins were crossed in all possible pairings and incubated at 25°C, 28°C and 30°C to evaluate the capacity to undergo teleomorph stage development. Significantly, after four weeks incubation cleistothecia were observed in one cross (65-14 x 65-16) at 28 °C and 30°C, and two crosses at 25°C among 18 crosses; all contained asci and ascospores when crushed. The cleistothecia formed along the barrage zones between isolates of opposite mating types and were fawn to dark brown or yellow in color (Figure 2 a-b).

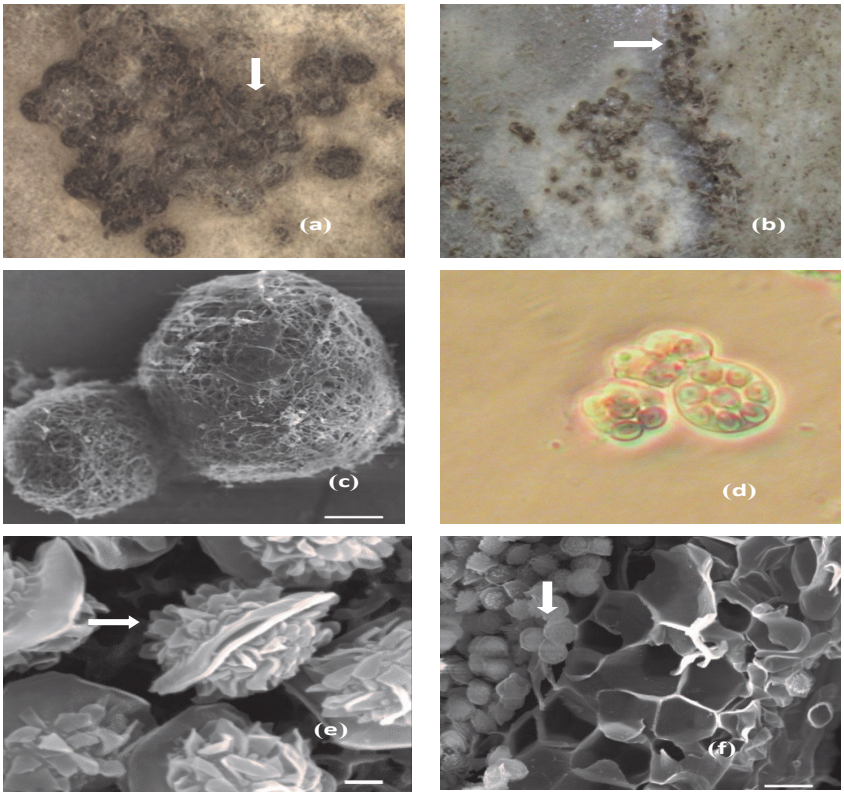

Figure 2: Sexual reproductive structures in A. clavatus, (a-b) Paired cultures of 65-14 x 65-16 on oatmeal agar with fawn to dark brown cleistothecia (arrowed) forming along the barrage zones following four weeks incubation at 25°C (c) SEM micrograph of a cleistothecium showing the interwoven hyphae that form the peridial wall. Scale bar, 100 µm.(d) a photomicrograph of an 8-spored asci.(e) SEM micrograph of lenticular ascospores (white arrow) Scale bar, 1µm. (f) Close-up of the peridium of interwoven hyphae with a group of ascospores, (white arrow). Scale bar, 10µm.

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After incubation for a further two weeks, the number of fertile crosses increased to six crosses at 25 °C, five crosses at 28°C, and just one cross at 30°C. Finally, after ten weeks of incubation, the number of fertile crosses increased to nine crosses at 25°C, (Table 3), remained at five crosses at 28°C , and just two (65-16 x 65-14; 65-16 x 65-8) crosses (11%) at 30°C. No additional cleistothecia were observed in any of the crosses at three incubation temperatures when cultures were re-examined after five months. Therefore, the longer incubation period of 10 weeks resulted in an increase in the number of fertile crosses, and among the possible 18 crosses, 50% were fertile at 25°C, which was found to be the best incubation temperature for A. clavatus of those assayed. Various other features of the crossing data were noteworthy. Firstly, mating was not observed in any crosses which involved an isolate from India (65-20). Secondly, where cleistothecia formed there was a large variation in isolate fertility depending on the mating partners (Table 3). The MAT1-2 isolate 65-16 was the most compatible with a range of different isolates. For example, the pairing 65-16 x 65-14 produced the highest number of cleistothecia at both 25°C and 28°C, and in a short period (~100 cleistothecia per Petri dish after four weeks). However, the same isolate 65-16 when crossed with 65-7 failed to produce cleistothecia, although the latter did cross with isolates 65-19 (Table 3). Also, there were relatively few cleistothecia produced overall, with 78% of the nine fertile crosses of A. clavatus producing less than 20 cleistothecia, and no crosses producing more than 100 cleistothecia under the incubation conditions. Thirdly, some crosses were more flexible in their temperature requirement for crossing than others, for example, isolates 65-2, 65-8, 65-10, and 65-14 produced cleistothecia at both 25 and 28 ºC, whereas isolates 65-7 and 65-18 were fertile at only 25 ºC. A formal description of the sexual state of A. clavatus is as follows. Cleistothecia superficial, subglobose to ovoid (315 - [513] - 692 µm), hard, yellowish-brown saffron colour (fawn), uniloculate, nonostiolate covered by dense aerial hyphae, maturing gradually from the centre outward after four weeks; covered by dense aerial hyphae stromatal peridium (57 µm). Asci 8 spored irregularly disposed globose to subglobose. Ascospores hyaline, lenticular (6.0- [6.5] - 7.0 µm), variously sculptured, with two equatorial crests (Figure 3).

Table 3: Number of cleistothecia produced by A. clavatus crosses on oatmeal agar medium at 25˚C in the dark after ten weeks.

Crosses		Number of cleistothecia MAT1-2		
		65-16	**65-19**	**65-20**
MAT1-1	65-2	+	+	-
	65-7	-	+	-
	65-8	+	-	-
	56-10	+	-	-
	65-14	+++++	+	-
	65-18	++++	+	-

Ratings indicate the mean number of cleistothecia produced from three replicate crosses on Oatmeal agar in 9cm diameter Petri dishes: -none; +, 1-19; ++, 20- 39; ++++ 60-79; +++++, 80-100; >, more than 100 cleistothecia.

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Evidence of recombination in ascospore progeny

To confirm that recombination had occurred in the ascospore progeny, it was first necessary to be able to produce pure suspensions of ascospores separate from contaminating asexual conidia. Spore suspensions containing both conidia and ascospores were heat treated at different temperature (68°C, 69°C, 70°C, 72°C, 75° C, and 80°C) for 10 min. However, it was found that the conidia and ascospores of A. clavatus were equally vulnerable to high temperature, unlike the situation in A. fumigatus and A. lentulus (Table 4). Thus, although ascospores of A. clavatus could survive heat treatment at 68°C for 10 min, so did conidia. Instead, a novel alternative method was developed. This involved heat treatment of intact cleistothecia at 69°C for 10 min before squashing the cleistothecia to release ascospores, from which single germinating ascospores were isolated as previously described. This experimental approach was found to heat inactivate any conidia in suspension while keeping the ascospores alive, presumably because the peridium of the cleistothecia served as a barrier to protect the ascospores to an extent from the heat shock. Notably, the cleistothecia were very hard relative to those of A. lentulus and A. fumigatus, and were, therefore, easy to clean by rolling on agar to remove adhering conidia. A representative gel of segregation patterns of the RAPD markers is shown in (Figure 3).

Table 4: Different temperature and time used to kill the conidia and ascospore for five isolates

	Aspergillus fumigatus	**Aspergillus lentulus**	**Aspergillus clavatus**	**Penicillium roqueforti**	**Penicillium paneum**
Conidia	80°C/ 30min	80°C/ 30min	69°C/ 10 min	70°C/ 10min	68°C/ 10min
Ascospore	90°C/ 60 min	90°C/ 60 min	*69°C/ 10min	75°C/ 10min	*68°C/ 10min

*The ascospore exposure to heat shock inside the cleistothecia.

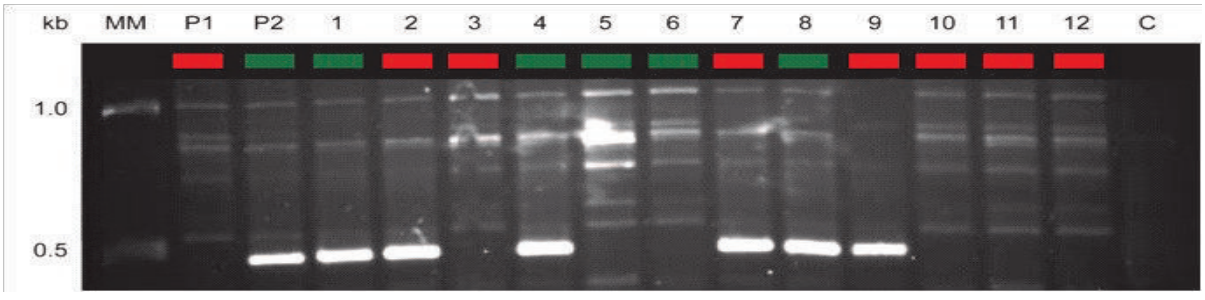

Figure 3.: Evidence for meiotic recombination, Segregation patterns of a RAPD-PCR amplicon in A. clavatus, parental isolates (P1, P2) and 12 ascospore progeny (1-12) from the cross 65-14 (P1) x 65-16 (P2), using primer OPC20. MM, molecular weight marker; C, water control; kb, kilobase. The arrow indicates the diagnostic RAPD band. Lane headings: MAT1-1 (red) and MAT1-2 (green) genotypes.

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An analysis of recombination among ascospore derived strains was then conducted to confirm that meiosis had taken place. There was clear evidence of recombination of genetic markers during the sexual cycle from the cross 65-14 x 65-16. Analysis of the progeny by PCR with mating-type specific primers revealed a near 1:1 segregation ratio of mating type [(MAT1-1:MAT1-2 = 6:6; χ^2=0.33; p=0.563, (p>0.05)]. Moreover, the RAPD analysis showed that the majority of these progeny had recombinant genotypes based on four RAPD-PCR markers. Combining the MAT and the RAPD data revealed that 83% of the progeny had unique genotypes (Table 5).

Table 5: Genotypes[a] in the parental isolates and 12 ascospore progeny of a cross between A. clavatus isolates 65-14 x 65-16.

Isolate	Mating type	RAPD band[b]				Genotype[c]
		OPC 20	OPT 18	UBC 90	OPQ6	
65- 14	MAT1-1	-	-	+	+	P1
65-16	MATI-2	+	+	-	+	P2
14-16-2	MAT1-2	+	+	-	-	A
14-16-4	MAT1-1	+	-	+	+	B
14-16-5	MAT1-1	-	+	+	-	C
14-16- 7	MAT1-2	+	+	-	-	D
14-16- 8	MAT1-2	-	+	+	+	E
14-16-9	MAT1-2	-	-	+	+	F
14-16-10	MAT1-1	+	-	-	-	G
14-16-11	MAT1-2	+	+	-	-	A
14-16-13	MAT1-1	+	-	-	+	H
14-16-14	MAT1-1	-	-	+	+	P1
14-16-18	MAT1-1	-	-	-	+	J
14-16-19	MAT1-1	-	-	+	+	P1
P- value(2- tailed)[d]		1.00	0.072	1.00	0.56	
Contingency χ^{2} [ef]			0.771 (1)			

[A] Genotypic characterization based on mating type and RAPD-PCR bands.
[b] RAPD-PCR bands amplified using Operon primers, OMT1 or R108 . '+' and' ⁻' denotes presence or absence, respectively, of particular amplicons.
[c] The genotype of each progeny isolate, defined by unique combinations of mating-type and RAPD markers as distinct from the parental isolates (designated P1 and P2), is identified by a different letter of the alphabet.
[d] Fisher's exact test for deviation from the null hypothesis of independent assortment of mating-type and RAPD markers in the progeny (i.e. 1:1:1:1 MAT1-1+ :MAT1-1- :MAT1-2+ :MAT1-2- ratio for each RAPD marker). Fisher's exact test was used instead of the χ^2 test because the expected frequencies were <5 (Spss software).
[e] To test for deviation from the null hypothesis of independent assortment of mating-type and RAPD markers in the progeny (i.e. an overall 1:1:1:1 MAT1-1+ :MAT1-1- :MAT1-2+ :MAT1-2-ratio for the sum of the RAPD markers).
[f] Number in parenthesis indicates the degree of freedom.

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Discussion

Previous phylogenetic work had revealed that the known sexual species Neocarpenteles acanthosporum and A. clavatus are clustered together [23]. This hinted at possible sexual reproduction in A. clavatus. Besides, one species in the section Clavati, Aspergillus ingratus, has been reported to produce saffron-colored sclerotia when in incubated in the dark.[24] Furthermore, a study [25] suggested a close relationship between N. acanthosporum and Neosartorya fischeri because both have hyaline oblate (bivaved) ascospores with distinct equatorial crests and variously sculptured convex surfaces. Moreover, the triangular projections from the ascospore wall of N. acanthosporum closely resemble the ascospore ornamentation of another Neosartorya species, N. pseudofischeri.[26] A survey of A. clavatus isolates from various worldwide locations was first conducted, using primer pairs.[16] This revealed the presence of both MAT1-1 and MAT1-2 isolates in a near 1:1 ratio, consistent with sexuality.[17] Therefore a series of sexual crosses were set up between known MAT1-1 and MAT1-2 isolates under a range of different conditions to see if sexual reproduction could be induced. It was shown that A. clavatus possesses a fully functional sexual reproductive cycle that results in the production of cleistothecia and ascospores, after four weeks incubation at 25˚C and 28˚C on Nescofilm- sealed oatmeal agar plates. The cleistothecia were fawn to brown/yellow in color similar to those of Ne-ocarpenteles acanthosporum in which the species produce hard and sclerotioid, fawn nonostiolate, unilocular stromata in which that take 3 to 4 weeks to mature and which contain hyaline ascospores that have two equatorial ridges.[14] Highest numbers of cleistothecia were found to develop in crosses incubated at 25°C. Cleistothecia were observed in some crosses after four weeks, but, an increased number of fertile crosses were observed when crosses were incubated for a total period of ten weeks. The ascospores of A. clavatus were found to be sensitive to heat shock treatment, unlike those of A. fumigatus and A. Lentulus.[19] Such temperature sensitivity is typical for spores of most aspergili e.g. thermal death times to kill conidia of A. parasiticus and A. flavus range from 8-59 sec at 60°C.[27] There was also variation in isolate fertility, the highest number of cleistothecia being formed in crosses between 65-16 and 65-14. However, 65-16 failed to produce cleistothecia when crossed with isolate 65-7, so fertility was dependent on the mating partner as previously discussed.[28] A recombinational analysis of the ascospores was then conducted to confirm that meiosis had taken place. Four stable RAPD markers and the MAT genotype were studied in 12 ascospore progeny and distinct segregation patterns were clearly observed. This provided clear evidence of a heterothallic sexual breeding system. Thus, this study demonstrates that A. clavatus, after being considered asexual since its discovery more than 40 years ago, can actually reproduce sexually.

Conclusion

The ability of A. clavatus to undergo sexual reproduction is highly significant in understanding the biology and evolution of the species. The presence of a sexual cycle provides an invaluable tool for classical genetic analysis and will facilitate research into the genetic basis of pathogenicity and fungicide resistance.

Conflicts of interest

The authors report no conflicts of interest.

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