A STUDY OF NATURAL POPULATION OF Neurospora
AND ISOLATION OF NOVEL MORPHOLOGICAL MUTANTS

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ABSTRACT

Neurospora is a tropical fungus which is found abundantly growing on burnt sugarcane, discarded corncobs and other burnt vegetation in India. We have studied the population of Neurospora at three different sites in Madhya Pradesh, India. These three sites were - Railway station, Ujjain, Bilavli Lake, Khandwa road, Indore and Sanver Road (Sanver road side, in front of Trimurti Restaurant). At these sites Neurospora is found growing on corncobs which are discarded after eating the roasted kernel in rainy season. The visual conidial samples of Neurospora and soil samples from these sites were collected and brought to the laboratory. From these samples 136 Neurospora cultures were isolated and purified. The mating types and species of representative of Neurospora cultures were determined. The study shows that the most common species is Neurospora intermedia but some cultures belonging to Neurospora crassa have been found in Sanver and Indore. The yellow ecotype of N. intermedia is predominantly present at all the three sites but, orange ecotype of N. intermedia is also found at Sanver and Indore. Both the mating types of Neurospora are equally prevalent at all the three sites. We have screened the isolated Neurospora cultures for morphological mutants. Ten cultures have been identified which show distinct morphological defects. The defects in the morphology have been characterized and are described in this paper.

Key Words: Neurospora, Neurospora intermedia, Natural population, Morphological mutants, Mating types, Yellow and orange ecotypes of Neurospora

INTRODUCTION

Neurospora is a filamentous fungus belonging to class Ascomycetes. It is commonly called red bread mold. In 1927, C. Shear and B. Dodge studied the sexual phase of this fungus and placed it in genus Neurospora.1 There are five conidiating species of Neurospora namely N. crassa, N. intermedia, N. sitophila, N. discreta and N. tetrasperma. N. tetrasperma is pseudohomothallic and other four species are heterothallic. Few homothallic species are also known but these are aconidiating.2 The work of Beadle and Tatum on nutritional mutants of Neurospora3 was one of the first studies that show direct relation between genetics and biochemistry. During the 20th century Neurospora research developed and the genus became an important model organism for the genetic, biochemical and cytological studies.4 Neurospora is one of the first fungal organisms whose genome was completely sequenced.5 On the other hand, even today the biology of this organism in nature is poorly understood and knowledge about its natural history is limited.6,7 In nature Neurospora is found in tropical and subtropical regions where it has been reported on materials such as on bread from bakeries,8 remains of burnt vegetation9,10, corn cobs11 and filter mud from sugar mills.11 It has also been reported on the burnt trees and shrubs in forests of Western North America and Southern Europe7 which have dry and cold climatic conditions. Natural distribution of Neurospora population differ from region to region12 however, all species have been reported from India13,14 including two different ecotypes of N. intermedia namely yellow ecotype and orange ecotype.6,15 Pinkish or salmon orange coloured N. intermedia

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AIMS AND OBJECTIVES

The goal of the study was to understand the Neurospora population at three different sites in Madhya Pradesh, India and compare them. Towards this the objectives of the study were to identify the most prevalent species and ecotype (yellow/orange) of Neurospora at these sites, and to determine the sexual or asexual nature of these populations by studying the distribution of different mating types. We also wished to screen the isolated Neurospora cultures for novel morphological mutants and purify them so that they can be used for understanding fungal growth mechanisms.

MATERIAL AND METHODS

Site selection

Three sites of Madhya Pradesh, India were chosen for the study and collection of Neurospora samples. The three sites were Railway station, Ujjain, Bilavli Lake, Khandwa road, Indore and Sanver Road side (in front of Trimurti Restaurant, Sanver). Sanver is located in between Ujjain and Indore, it is about 25 km away from Ujjain and about 35 km away from Indore. These sites were chosen because at all these three sites roasted corns are sold regularly and cobs are discarded after eating roasted kernel. Patches of orange or yellow coloured Neurospora growing on discarded corncobs can be easily seen during rainy season at these three sites.

Collection of samples

From each site soil as well as conidial samples were collected during June 2009 to April 2012. Soil samples were collected from various places during summer season in sterilized polythene bags and stored at room temperature. Conidial samples were collected from visible growth patches of Neurospora present on discarded corn cobs during rainy season by using the method described by Perkins and Turner. These samples were stored in refrigerator.

Isolation and purification of Neurospora

For isolation of Neurospora cultures the filter paper strips containing conidial samples were aseptically placed on the surface of Vogel’s minimal agar medium containing 0.02% chloramphenicol. The plates were incubated for 24 hr at 34±2°C. The conidia germinated and formed vigorously conidiating cultures. The fresh conidia were transferred to Vogel’s agar slants. Neurospora cultures were also isolated from soil using the modified method of Maheshwari and Antony. The cultures were purified by repeated subculturing.
Mating type determination and species identification

The mating types of *Neurospora* cultures were determined by spot crosses on lawn of fluffy tester strains as described by Perkins et al. Species of the isolated *Neurospora* cultures were determined by testing the fertility in the crosses with tester strains as described by Perkins and Turner. Isolates were crossed with tester strains of different species obtained from Fungal Genetic Stock Centre (FGSC), Kansas City, USA. Each *Neurospora* isolate was crossed with all the four tester strains [N. crassa, 74-OR23-IVA (FGSC 2489 mat A) and OR56a (FGSC 4200 mat a), N. intermedia, (FGSC 1766 mat A and FGSC 1766 mat a), N. sitophila (FGSC 2216 mat A and FGSC 2217 mat a), N. discreta (FGSC 3228 mat A and FGSC 3229 mat a)] and the cross tubes were observed between 10-15 days. The *Neurospora* isolates produced fertile perithecia containing abundant ascii and shot black ascospores on the walls of the cross tube only with the tester strain of its own species. If a culture is crossed with tester strain of the same species then, it is fully fertile producing beaked perithecia and abundant ascospores ( > 50% black and viable) but, if the culture is crossed with tester strain of other species then it produces rudimentary perithecia which eject few pigmented and viable ascospores.

Morphological studies

For studying the morphology the cultures were grown on petri dishes containing Vogel's minimal media and incubated at 34±2°C. The colour of the conidia of the cultures was visually recorded and conidial size was determined using micrometer. The extension growth rate was determined by growing the cultures in Race tubes. The colony characteristics were recorded and details of hyphal growth were observed under microscope (40X, 100X and 400X magnification).

Inheritance of the morphological defects

To study the inheritance of morphological defects the mutants were crossed with tester strains obtained from FGSC [N. intermedia, (FGSC 1766 mat A and FGSC 1767 mat a)] and N. intermedia strain (RM126-3A obtained from R. Maheshwari). Twenty viable progeny from each cross were screened for morphological defects to determine if they inherited the parental defect or not. To determine cytoplasmic (plasmid/mitochondrial) or nuclear basis of inheritance reciprocal crosses were made.

RESULTS AND DISCUSSION

Railway station, Ujjain, Bilavli Lake, Khandwa road, Indore and Sanver Road (Sanver road side, in front of Trimurti Restaurant, Sanver) were the sites selected for the study of *Neurospora* population. At these sites vendors regularly sell roasted corns. The sites were regularly visited and the discarded corn cobs were examined for the presence of *Neurospora*. Our observations show that *Neurospora* appears as profusely conidiating patches on cobs during the entire rainy season (June to August in India) (Fig. 1(a) and 1(b)). The colour of the *Neurospora* patches is either saffron yellow or salmon orange (Fig. 1(a) and 1(b)). The visible conidial samples were collected during rainy season on sterile filter paper strips and brought to the laboratory for isolation of cultures. *Neurospora* cultures were also isolated from soil samples which were collected from all the three sites during summer season. In total 136 *Neurospora* cultures were isolated from soil and conidial samples. The cultures were purified and their mating types were determined (Table 1). The colour of conidia was visually recorded. The results are summarized in Table 1. The species of few representative cultures from each site was determined by crossing the cultures with tester strains of different species. The results show that from Sanver road side, total 44 *Neurospora* cultures were isolated out of these 34 were saffron yellow in colour and 10 were salmon orange coloured (Table 1). Conidial size of representative saffron yellow and salmon orange cultures was measured. Conidial size of saffron yellow coloured cultures was 11.4-20 µm whereas the conidial size of salmon orange coloured cultures was 5.72-8.57µm. Species of ten representative cultures was determined. The results show that all the saffron coloured cultures belong to *N. intermedia*, whereas salmon orange coloured cultures belong either to *N. crassa* or *N. intermedia*. As described by Perkins and Turner the *N. intermedia* cultures having saffron yellow coloured conidia of large size belong to distinct
ecotype which they called yellow ecotype whereas salmon orange coloured cultures of \textit{N. intermedia} having small sized conidia belong to orange ecotype.

\textbf{Fig.1(a)} : \textit{Neurospora} growing on discarded corncobs, saffron yellow coloured conidiation

\textbf{Fig.1(b)} : Salmon orange coloured conidiation

\textbf{Table 1 : Variation in conidial colour and distribution of mating types in Neurospora population at three different sites in Madhya Pradesh, India}

\begin{table}[h]
\begin{center}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
S/N & Site of sample collection & Total no. of cultures isolated & Saffron Yellow cultures & & Salmon Orange cultures & & \\
& & mat ‘A’ & mat ‘a’ & Total & mat ‘A’ & mat ‘a’ & Total \\
\hline
1 & Sanver Road side & 44 & 14 & 20 & 34 & 6 & 4 & 10 \\
2 & Bilavli Lake, Indore, India & 62 & 19 & 29 & 48 & 5 & 9 & 14 \\
3 & Railway Station, Ujjain, India & 30 & 12 & 18 & 30 & – & – & – \\
\hline
\end{tabular}
\end{center}
\end{table}

Thus yellow ecotype of \textit{N. intermedia} is predominantly present at this site as 77\% of the cultures had saffron yellow coloured large sized conidia. At this site orange ecotype of \textit{N. intermedia} having salmon orange coloured small size conidia is also found, although it is present in very small number. \textit{N. crassa} forms the minor component of the population. Mating type analysis showed that out of 34 saffron yellow cultures 14 were of mating type ‘A’ and 20 were of mating type ‘a’. Out of 10 salmon orange cultures 6 were of mating type ‘A’ and 4 were of mating type ‘a’. From Bilavli Lake situated at Khandwa road, Indore 62 \textit{Neurospora} cultures were isolated and among these cultures 48 were saffron yellow coloured and 14 were salmon orange coloured (\textbf{Table 1}). Out of 48 saffron yellow coloured cultures 19 were of mating type ‘A’ and 29 were of mating type ‘a’ and

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out of 14 salmon orange cultures 5 were of mating type ‘A’ and 9 were of mating type ‘a’ (Table 1). Species of ten representative cultures were determined and their conidial size was measured. The results show that at this site also the predominant species is yellow ecotype of *N. intermedia* which forms about 77% of the population. At this site also, few cultures of orange ecotype of *N. intermedia* as well as *N. crassa* were found which form minor component of the population. From railway station, Ujjain 30 *Neurospora* cultures were isolated. Species of four representative cultures were determined. All the cultures had saffron yellow coloured conidia (Table 1). Conidial size of representative cultures was measured and it was 11.4-20 μm. Thus all isolated cultures from railway station, Ujjain belong to yellow ecotype of *N. intermedia*. The mating type analysis showed that in the population 18 *Neurospora* cultures were of mating type ‘a’ and 12 cultures were of mating type ‘A’. Thus it appears that at this site *Neurospora* population is consists of yellow ecotype of *N. intermedia*. However, previous studies had shown occurrence of few salmon orange cultures at this site (personal observations of authors).

Thus it appears that at all the three sites in Madhya Pradesh, India yellow ecotype of *N. intermedia* is predominantly present and minor component of the population is represented by *N. crassa*. At all the three sites both the mating types are equally distributed which indicates that besides asexual reproduction by conidiation the population may also be reproducing sexually. Interestingly, few cultures of orange ecotype of *N. intermedia* have also been found. This is in contrast with *Neurospora* population in Maddur found on fire-scorched sugarcane (Karnataka, India), where 95% of the population consists of orange ecotype of *N. intermedia* and rest of *N. tetrasperma*. This population is also reproducing sexually. Our results corrobo-rate with the studies of Perkins et al., Parkins and Turner and Turner et al., who reported that *N. intermedia* is the predominant species in many areas around the world. However, it is interesting to note that at all these sites in central India in present study the predominant species is the yellow ecotype of *N. intermedia* whereas in south India the predominant species is orange ecotype of *N. intermedia*. The yellow ecotype has previously been reported from the Eastern Hemisphere except for one strain from Hawaii. Our observations also confirm that orange ecotype is sometime present along with yellow ecotype on discarded corncobs. The population structure of central India is different from other places in the world for example in Western North America *N. discreta* is the most frequent species and forms 95% of the *Neurospora* population. In southern Europe *N. crassa* and *N. sitophila*, are more frequent whereas in Spain *N. crassa*, and *N. discreta*, are more frequent. Europe and Western North America are temperate regions of similar latitude, climate and vegetation and absence of *N. intermedia* in these regions suggests the possibility that *N. intermedia* is not adapted to cold and/or dry climatic conditions of temperate regions. Based on these studies we can say that the species diversity of *Neurospora* differs in various regions globally.

**Morphological mutants**

We have screened 33 cultures isolated from above sites for morphological defects. The cultures were grown in petri plates containing Vogel’s minimal medium and were observed under microscope. We selected 10 cultures showing distinct morphological defects for further study. The details of morphological defects were characterized and their growth rates were measured using race tubes. Fig. 2 shows the growth curves of these mutants. It is clear from these graphs that all these are slow growing mutants. Fig. 3 shows the culture morphology in Petri dish and Fig. 4 shows morphological defect under microscope. The defects in the morphology and growth rates are described in Table 2.
Our results show that out of 33 *Neurospora* culture selected for morphological studies 10 had distinct morphological defects. Thus about 30% of the population shows defect in fungal morphology. We have characterized the defects in 10 morphological mutants which are shown in Table 2 and Fig. 3 and Fig. 4. All the defective cultures had reduced growth rates which are shown in Fig. 2. The reduction in growth may be due to defective branching, loss of polarity in tip growth or other hyphal growth abnormalities. We have found mutants which show branching defects like, hyperbranching (I9-19 and I9-29), branching at tip and dichotomous branching (S9-12, U10-13, I9-19 and I9-20), reduced frequency of branching (I9-33 and U10-15) and reduced branch growth and no branching in lateral branches (S9-5). We have also found mutants showing defects in tip growth which causes loss of polarity (S9-4, I9-20 and S9-12) and mutant showing defect in hyphal growth like meandering of hyphae (S9-5). Many mutants had reduced aerial branching and conidiation Table 2.

Previous studies have reported that about half of the stains of *Neurospora* collected from nature contain plasmids. The exact effect of these plasmids on the stains is not clear but many of them leads to fungal senescence.44,45

We wanted to see if the mutants isolated by us had morphological defects due to cytoplasmic (plasmid) or nuclear gene. We selected three morphological mutants for this study (U10-18, I9-20 and I9-29). We made reciprocal crosses of these cultures with FGSC testers to test the cytoplasmic (plasmid) or nuclear basis of these defects. The details of the reciprocal crosses of three mutants are shown in Table 3. From each cross after one month random ascospore analysis was done. Twenty viable progeny from each cross was studied to see the inheritance of the parental defect in the progeny Table 3. The growth rates and morphology of each of the progenies were compared with the respective wild type and mutant parents. The results shows that only few progeny resembled the mutant parent with respect to both growth rate and hyphal morphology Table 3. Few progenies have wild type growth rates but have morphological defect similar to mutant parent showing that there is no relation between growth rate and morphological defect. The results of reciprocal crosses are similar which clearly points out that the defects are due to nuclear gene and not due to some cytoplasmic factor.
Fig. 3: Morphology of wild type and morphological mutant strains of *Neurospora* growing on Vogel’s minimal agar media at 34±2°C temperature. (a) Wild type (*N. intermedia*, FGSC 1767), (b) Wild type (*N. crassa*, FGSC 4200), (c) I9-29, (d) I9-33, (e) S9-4, (f) U10-15, (g) S9-12, (h) U10-13, (i) I9-19, (j) U10-18, (k) I9-20 and (l) S9-5
Table 2: Morphological defects in *Neurospora* mutants isolated from nature and their growth rates

<table>
<thead>
<tr>
<th>S/N</th>
<th>Culture No.</th>
<th>Morphological defects</th>
<th>Extension growth rate at 34 ±2°C (mm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wild Type N.intermedia (FGSC-1767a) (Fig. 3(a) and Fig. 4(a))</td>
<td>Fast growing. Salmon orange coloured conidiation. Hyphae grow straight. The hyphae branch periodically, mainly lateral branches are formed.</td>
<td>4.7</td>
</tr>
<tr>
<td>2</td>
<td>I9-29 (Fig.3(c) and Fig. 4(c))</td>
<td>Reduced growth. Saffron yellow coloured conidiation. Conidiation rhythm is seen. Surface conidiation present all over the culture with bands. Tips of the hyphae bends at places. Erratic branches and branching starts at 90 degree angle but later on moves ahead. The base of branch swollen. At certain places hyperbranching occurs at the tip forming a bunch.</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>I9-33 (Fig. 3(d) and Fig.4(d))</td>
<td>Extremely reduced growth. Saffron yellow coloured conidiation. Poorly conidiating, conidiation in rhythm. Very short aerial hyphae. Branching reduced and almost at right angles. The point from where branch arises becomes swollen. At the time of conidiation the aerial hyphae becomes thin and stop growing.</td>
<td>0.21</td>
</tr>
<tr>
<td>4</td>
<td>S9-4 (Fig. 3(e) and Fig. 4(e))</td>
<td>Thick mycelium in the middle of the colony with reduced yellow coloured conidiation. Reduced growth and aerial hyphae. Branching angle increased. The tips lose polarity and bends. The side branches are short and branch infrequently. The distance between the branching is erratic. Frequency of branching at one side is more.</td>
<td>0.62</td>
</tr>
<tr>
<td>5</td>
<td>U10-15 (Fig. 3(f) and Fig. 4(f))</td>
<td>Reduced conidiation. Saffron yellow coloured conidiation. In the colony growth stops and few hyphae continues growth and flares up at certain points. Therefore the margin of the colony is uneven. Some hyphae curl at the tip which may lead to stopping growth at certain points. On resuming growth hyphae have very few and short branches. Reduced and short branching. After some growth the mycelia forms dark brown coloured mat and uneven colony margin. Few hyphae extend beyond rest.</td>
<td>0.42</td>
</tr>
<tr>
<td>6</td>
<td>S9-12 (Fig. 3(g) and Fig. 4(g))</td>
<td>Saffron yellow coloured conidiation in patches. The culture forms thick mycelial mat. Rhythm is seen. Growth stops and then resumes at certain points and flares up. Reduced branching. Thin mycelium. Loss of polarity in tips region. Swelling at the base of branching. At certain points angle of branching increases and approaches almost 90 degrees. Branching starts very near to the tip but distance between branches are erratic (sometimes very small, sometimes very large). At few places dichotomous branching is also seen.</td>
<td>0.33</td>
</tr>
<tr>
<td>No.</td>
<td>Reference (Figures)</td>
<td>Description</td>
<td></td>
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<tr>
<td>7</td>
<td>U10-13 (Fig. 3(h) and Fig. 4(h))</td>
<td>Saffron yellow coloured conidiation. Initially the culture grows as circular colony but latter the growth stops and initiates at certain points in fan like pattern and forms circular margin of colony. The conidiation rhythm is seen. The center of colony has thick mycelium. Reduced aerial hyphae and conidiation in center of the colony. In the later period of growth the conidiation increases and growth occurs in a distinct pattern. Initially the fungal hyphae are thin having reduced branching with short hyphae. At places the tip loses polarity and bend but when growth resumes again then fungus grows in a fan like shape having slightly thicker hyphae and dichotomous branching pattern. The fungus shows dichotomous branching less frequently in initial culture but this type of growth is predominantly seen after the culture resume the growth. The distance between branches is extremely reduced and branching occurs at tip. High branching frequency. Swollen tips. Produce flavor (fruity odor)</td>
<td>0.25</td>
</tr>
<tr>
<td>8</td>
<td>19-19 (Fig. 3(i) and Fig. 4(i))</td>
<td>Reduced growth. Saffron yellow coloured conidiation. Thick mycelium at certain places. Erratic branching, dichotomous at tip but later one branch increases in length due to which branching appears alternate. At some points hyperbranching at tip. At some places distance between branching erratic.</td>
<td>1.1</td>
</tr>
<tr>
<td>9</td>
<td>U10-18 (Fig. 3(j) and Fig. 4(j))</td>
<td>Saffron yellow coloured conidiation. The growth stops and resume at tip. Base of branching is swollen. After dichotomous branching, one branch becomes short and one long. So in later part of culture branching appears alternate at some places but at other places bunch of branches is seen.</td>
<td>0.42</td>
</tr>
<tr>
<td>10</td>
<td>19-20 (Fig. 3(k) and Fig. 4(k))</td>
<td>Blackening of hyphae occurs as the fungus grows. Saffron yellow coloured conidiation. Surface conidiation and conidiation rhythm is seen. Irregular branching. Tip of hyphae lose polarity and enters into agar. At many places branching is dichotomous and at right angles. The base of the branch point is swollen. Sometimes the hyphae cannot grow straight and takes undulating growth. Curling of tips occurs at few places.</td>
<td>0.25</td>
</tr>
<tr>
<td>11</td>
<td>S9-5 (Fig. 3(l) and Fig. 4(l))</td>
<td>Slow growth. Conidiating having saffron yellow conidiation. Fungus forms thick mycelial mat. Hyphal growth stops and then flares up at certain points. Rhythm seen. Defective branching. During initial growth the branching angle is increased and branching distance is decreased. The lateral branches are short and show infrequent branching or remain unbranched. In later phases at certain places dichotomous branching is also seen and at some points the base of the branching becomes swollen. The hyphae do not grow straight but takes an undulated pattern.</td>
<td>0.42</td>
</tr>
</tbody>
</table>
Fig. 4: Morphology of wild type and morphological mutant strains of *Neurospora* at 34°C temperatures. Fungi were grown on Vogel’s minimal agar medium for 24h and images were taken at 40X magnification. (A) Wild type (*N. intermedia*, FGSC#1767), (B) Wild type (*N. crassa*, FGSC#2489), (C) I9-29, (D) I9-33, (E) S9-4, (F) U10-15, (G) S9-12, (H) U10-13, (I) I9-19, (J) U10-18, (K) I9-20 and (L) S9-5. Bars are 100μm.
<table>
<thead>
<tr>
<th>S/N</th>
<th>Cross number and parents</th>
<th>Ascospore viability (% germination)</th>
<th>Analysis of 20 viable progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cultures having growth rates similar to wild type</td>
</tr>
<tr>
<td>1</td>
<td>AM25 N. intermedia (1767) [♀] X U10-18 [♂]</td>
<td>59%</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>AM26 U10-18 [♀] X N. intermedia (1767) [♂]</td>
<td>20%</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>AM71 N. intermedia (RM126-3A) [♀] X I9-20[♂]</td>
<td>30%</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>AM81 I9-20[♀] X N. intermedia (RM126-3A) [♂]</td>
<td>55%</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>AM45 N. intermedia (1767) [♀] X I9-29[♂]</td>
<td>19%</td>
<td>18</td>
</tr>
<tr>
<td>6</td>
<td>AM46 I9-29 [♀] X N. intermedia (1767) [♂]</td>
<td>31%</td>
<td>17</td>
</tr>
</tbody>
</table>

**CONCLUSION**

Thus we have purified the mutants which have heritable defects. Further studies are needed to test and identify the genes responsible for these defects and to find the exact position of gene on the chromosomes by classical genetic mapping. All these are mutants of *N. intermedia* so we are trying to introgress these genes in the *N. crassa* so that above mentioned studies can be done. These mutants may be useful for understanding the mechanism involving fungal growth and branching.

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