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PARASEXUAL ANALYSIS OF *Aspergillus awamori* BY USING INTRASPECIFIC DIPLOIDS AND INTERSPECIFIC HYBRIDS WITH *A. niger*

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ABSTRACT

Auxotrophic, morphological and resistant mutants have been isolated from *Aspergillus awamori* NRRL 3112 strain and used for conventional genetical analysis by the parasexual cycle. A minimum of four linkage groups was suggested for that species. Also interspecific hybrids with *A. niger* were obtained only after protoplast fusion and segregants from them suggested: I) that genetical analysis should be done as there is confirmation of linkage groups previously determined with intraspecies diploids; II) new linkage relationships between *awamori* markers and III) homology of at least one linkage group on both species that allowed mitotic crossing over to occur. These suggestions are considered as indications of a very close phylogenetical relationship between these species.

INTRODUCTION

The filamentous fungus *Aspergillus awamori* is of considerable biotechnological interest mainly because it is used for the industrial production of glucoamylase (Banks *et al.*, 1976; Fogarty and Kelly, 1980). Although several studies have been made of fermentation and of molecular aspects of enzyme production with this species (Nunberg *et al.*, 1984; Bhella and Altosaar, 1988; Hayashida *et al.*, 1988)

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very little work has been done on it's genetics, probably because of the genetical instability of some of the available strains. Vialta and Bonatelli Jr. (1988) detected spontaneous segregation of conidia and formation at a high frequency of sectors with a requirement for proline (*pro* A_1) in *A. awamori* NRRL 3112. They also reported that in spite of several attempts, they were not able to isolate fully stable *pro* A^+ strains and that *pro* A_1 colonies were extremely stable (reversion frequency < 10^{-8}). A tentative explanation for that situation was a chromosomal instability involving a segment including part of, or within, the *pro* A^+ gene, that when excised generates essentially stable *pro* A_1 derivatives.

There is one report indicating that the parasexual cycle exists in *A. awamori* (Ogawa *et al.*, 1988a). These authors used two single auxotrophic mutants isolated from *A. awamori* var. *kawachi* and obtained heterokaryons by using protoplast fusion techniques. They isolated putative heterozygous diploids after *d*-camphor treatment which they characterized by nuclear DNA content, conidial diameter and spontaneous segregation of auxotrophic markers. No clear relationship of linkage could be established, probably because few genetic markers were used and also because the identification of segregants was apparently time consuming as conidial color marker(s) were not utilized.

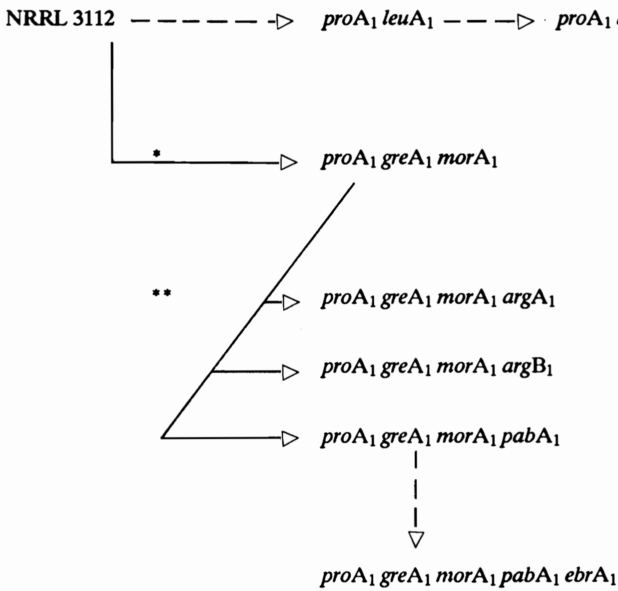
In this paper we report the isolation of auxotrophic, morphological and resistant mutants from *A. awamori* NRRL 3112 that have been used for detecting the existence of the parasexual cycle in this strain. We chose this strain because it has been used in several research programs aiming at isolating improved glucoamylase producing strains and also because the glucoamylase gene has been cloned (Park and De Santi, 1977; Nevalainen and Palva, 1979; Nunberg *et al.*, 1984).

MATERIAL AND METHODS

Strains

It was used the *A. awamori* NRRL 3112 strain from NRRL, Peoria, USA. Auxotrophic, morphological (for conidial color or colony form) and resistant mutants of two *pro* A_1 (proline requiring) derivatives of the parental strain were induced with short-wave ultraviolet light irradiation at a survival level of 1-5% (Vialta and Bonatelli Jr., 1988). All but one of the auxotrophic mutants were isolated after filtration enrichment (Fries, 1947). For each gene, three-letter symbols were used (see Table I). The *A. niger* strain used was *pab*A_1 *nic*A_1 *olv*A_3 (*p*-aminobenzoic acid and nicotinic acid requiring with olive conidial colour) obtained as a segregant from the diploid *nic*A_1 *olv*A_3//*pab*A_1 *fwn*A_1 (Bonatelli Jr. *et al.*, 1982, 1983).

Table I - Auxotrophic, morphological and resistant mutant strains isolated from *A. awamori* after UV irradiation with (continuous lines) or without filtration (dashed lines).



Gene symbols: *arg*A$_1$, *arg*B$_1$, *leu*A$_1$, *pab*A$_1$, and *pro*A$_1$ are nutritional requirements for arginine, arginine (or citrulline), leucine, *p*-aminobenzoic acid and proline (or arginine, or citrulline or ornithine) respectively; *bwn*A$_1$ and *gre*A$_1$ are brown and green conidial colors; *mor*A$_1$ is thin mycelium; and *ebr*A$_1$ is for resistance to ethidium bromide.

* One filtration step; ** two filtration steps.

Media

Culture media for vegetative growth were essentially those described by Pontecorvo *et al.* (1953b). Minimal medium (MM) was supplemented as necessary with vitamins (1-5 μg/ml) and aminoacids (50-100 μg/ml). For resistant mutant isolations, ethidium bromide (EB, Sigma) was added at a final concentration of 24 μg/ml of complete medium (CM). Dose response experiments with sensitive strains, resistant mutants and heterozygous diploids were done with EB concentrations up to 85 μg/ml of CM. For protoplast fusion experiments, MM and CM were prepared at two-fold concentrated and mixed with 1.2 M KCl solution pH 5.8 just before use to have a proper final concentration of nutrients and KCl. Incubation temperature was 28-30°C in all experiments.

Intraspecific crosses

Heterokaryons of *A. awamori* were obtained by using Roper's technique (1952) with minor modifications (Bonatelli Jr. *et al.*, 1983). Conidia were collected in Tween 80 solution (0.1%, v/v) from heterokaryons maintained on MM plus proline and 10^4 to 10^6 were plated per petri dish containing MM plus proline. After 4-7 days of incubation conidia from putative diploid colonies were collected (see above) and up to 200 were plated on petri dishes with CM or MM plus proline. If the number of colonies formed was the same on both media, one isolated colony was further purified by streaking conidia on MM plus proline. After these two steps of purification, slants of MM plus proline and CM were prepared, incubated up to 5 days and stored at 4°C. These steps were necessary because of the non-autonomous conidial color of the heterokaryotic colonies (Pontecorvo *et al.*, 1953a) and also because the latter were 1.2 to 38 times more frequent than putative diploid colonies. Diploid colonies were characterized by conidial diameter, number of nuclei per conidium, sensitivity to the benlate test (Upshall *et al.*, 1976), segregation on benlate containing CM (Hastie, 1970) and DNA content per nucleus (Giles and Myers, 1965; Davidse, 1973). Haploidization on benlate-containing CM was better when the concentration was 1-2 μg/ml (0.5-1.0 μg of active principle). When benlate was dissolved only in acetone there was a significant decrease of sectors (Vialta, 1987) so, benlate was first dissolved in up to 1 ml of acetone and then distilled sterilized water was added with occasional agitation to a final volume of 100 ml. That suspension was vigorously shaken just before use and aliquots were taken immediately after complete sedimentation. Segregants were classified for ploidy by conidial diameter and sensitivity to the benlate test (see above) before nutritional tests on supplemented minimal medium.

Protoplast fusion and interspecies hybridization

Protoplasts were obtained from *A. awamori* and *A. niger* strains by using the technique of Hamlyn *et al.* (1981). At least 10^6 of each strain were mixed and fused (Anné and Peberdy, 1976) and added to molten MM or CM with or without 0.6 M KCl (see above), mixed thoroughly and after media solidification incubated up to 15 days. Putative heterokaryons were transfered to fresh MM and/or CM by taking small pieces of mycelium developed on MM plus 0.6 M KCl. That procedure was repeated until conidiophores showing wild type conidial colour were observed and these were streaked on MM and CM as a first step of purification and to check nutritional requirements, if any. Colonies growing vigorously on MM were further purified by plating up to 100 conidia on MM and CM. These procedures were necessary because of the non-autonomous conidial colour of heterokaryons (Oliveira and Bonatelli Jr., not published). After that, putative interspecies hybrids were classified in the same way as *A. awamori* diploids (see above). Segregants were easily isolated and after purification they were classified for ploidy by the same procedure used for intraspecies segregants and assayed for nutritional requirements. As *A. awamori* is able to form a starch hydrolysis halo (*shh*) on MM containing starch as the sole carbon source and *A. niger* is not, interspecies hybrid and segregants were also tested for that phenotype. All of them had their starch index (halo diameter divided by colony diameter) determined.

RESULTS AND DISCUSSION

Mutant isolations

Two *pro*A_1 derivatives were chosen for mutant induction (Table I). The only auxotrophic mutant isolated without filtration enrichment was *pro*A_1 *leu*A_1 after assaying 1,200 surviving colonies. Because of the very low frequency (0.08%) filtration enrichment was used. One filtration step seemed not to be enough as 800 colonies yielded only one double morphological mutant (Table I). However after two filtration steps 3 auxotrophic mutants were isolated out of 286 colonies (1%). That frequency corresponds to an increase of 12.5 times over total isolation and is similar to reports on literature (Silveira and Azevedo, 1984). We propose that the low frequency observed when total isolation was used can be attributed, at least in part, to the frequency of conidia with 2 or 3 nuclei (42%) that might have hampered the isolation of auxotrophic mutants which normally are recessive especially because the multinucleated conidia should be more resistant to mutagenic treatments. Considering the evidence that two steps of filtration may be necessary for isolation of auxotrophic mutants it is probable that most of the prototrophic or partially prototrophic conidia are still not fully germinated, e.g., retained by gauze, after 24 h of growth on minimal medium plus proline.

The *leu*A_1 strain exhibited a crinkled colony morphology, a lower growth rate (about 2/3 of the parental strain) and conidia were formed later than in other strains. A morphological character - *mor*$_2$ - co-segregated consistently with leucine deficiency, suggesting a pleiotropic effect. Another pleiotropic effect of the *leu*A_1 mutation was that it seemed to dilute conidial colour, especially when combined with the *bwn*A_1 gene. A similar effect on conidial colour was reported for the *arg*B gene of *Aspergillus nidulans* (Upshall, 1986).

Ethidium bromide (EB) resistant mutants were isolated at a frequency of 2.5 x 10^{-5} only after UV induction. When untreated conidia were plated (10^6 to 10^7/petri dish) on CM plus EB, two types of colonies appeared and neither of them were able to grow when sub-cultured on fresh medium plus EB, even at lower drug concentrations. This has also been observed when mutants resistant to EB are isolated from *A. nidulans* (Scarazzatti *et al.*, 1979; Scarazzatti-Frau, 1985), especially when spontaneous mutants are selected. One of these "pseudo-resistant" colonies was crossed to a sensitive one via the sexual cycle and there was no indication of a suppressor gene or segregation of lower level resistant strains. One could speculate that, as in the case of *A. nidulans*, the heavy innoculum created a microenvironment that allowed growth and formed "pseudo-resistant" colonies.

A pleiotropic effect was also detected for the *gre*A_1 gene in a way similar to the *olv*A_1 gene in *A. niger* (Bonatelli Jr. *et al.*, 1985) that showed phialide proliferations. Co-segregation was consistently observed for these two markers.

Intraspecific crosses

Heterokaryons grew only when plated on MM plus proline, indicating that the same gene was affected in all strains. The same was observed for heterozygous diploids that had been isolated from all the crosses made (Table II). The frequency of putative diploid colonies varied from 1.7×10^{-6} to 7.1×10^{-5}. The higher frequency was obtained when mixed growth mycelium was maintained on liquid MM plus 4% of CM for 10 days instead of 4 to 5 days. These frequencies are lower than those reported by Ogawa *et al.* (1988a) because they cultivated heterokaryons on MM plus *d*-camphor. One putative diploid colony was chosen from each synthesized heterokaryon and characterized (Table II). They showed the same response to the benlate test (Upshall *et al.*, 1976); a conidial diameter 1.2 to 1.3 times of that observed for haploid strains, and that the percentage of multinucleated conidia is not a valid criterium for diploid characterization. One diploid strain had its DNA content per nucleus determined and it was 1.7 to 1.9 times greater than the haploid content, similar to the values obtained when DNA was extracted and/or quantified by other methods and even in other organisms (Heagy and Roper, 1952; Messias and Azevedo, 1980; Ogawa *et al.*, 1988a).

Dose response experiments showed that a diploid heterozygous for the *ebr*A_1 gene had a 50% reduction in colony size at 80 μg of EB/ml of CM, which is almost the same as in a resistant haploid strain (82 μg/ml), indicating that the characteristic is dominant. Similar results have been reported for some *A. nidulans* mutants although they are resistant only up to 30 to 50 μg/ml (Scarazzatti-Frau, 1985). High levels of resistance to that drug were also found in mutants isolated from the *A. niger* strain refered to in this work (Masiero and Bonatelli Jr., 1989).

Spontaneous and induced segregants were isolated from the diploid 1//2 (Table II). Although only 40 diploid colonies growing on CM were inspected, all the 25 segregants had a brown conidial colour and 40% of them were classified as diploids according to the benlate test and conidial diameter. In contrast, from 80 colonies point innoculated on CM plus benlate, 69 haploid segregants (37 brown and 32 green) were isolated. From these results it seems that green segregants are more difficult to isolate as spontaneous sectors and also that benlate is an efficient haploidizing agent for this species, as has been demonstrated for several other filamentous fungi (Hastie, 1970; Upshall *et al*., 1976; Bonatelli Jr. *et al*., 1983). These induced haploid segregants and others isolated from the three heterozygous diploids (Table II) were classified for nutritional requirements and morphology (Table III). As above the *leu*A_1 gene co-segregated with *mor*$_2$ 243 sectors and the same was true for *gre*A_1 and phialide proliferation. Taking these considerations and the results shown in Table III into account the following linkage groups could be suggested:

Group I - *gre*A_1 *bwn*A_1 *ebr*A_1
Group II - *mor*A_1 *arg*A_1 *arg*B_2
Group III - *leu*A_1
Group IV - *pab*A_1

Table II - Response to benlate, conidial diameter, DNA content per nucleus and percentage of multinucleated conidia (2 and 3 nuclei) of parental, mutant and diploid strains derived from heterokaryons synthesized between mutant strains of *A. awamori*.

	Response to benlate ($2\ \mu g\ ml^{-1}$)*	Conidial diameter (μm)	Multinucleated conidia (%)	DNA per nucleus ($\mu g \times 10^{-8}$)
Parental wild type				
NRRL 3112	R	4.8	42	4.3
Mutant strains				
1. *pro*A_1 *leu*A_1 bwnA_1	R	4.9	47	3.9
2. *proa*A_1 *pab*A_1 *mor*A_1 *ebr*A_1 *gre*A_1	R	4.8	41	ND
3. *pro*A_1 *arg*A_1 *mor*A_1 *gre*A_1	R	4.8	40	ND
4. *pro*A_1 *arg*B_1 *mor*A_1 *gre*A_1	R	4.8	44	ND
Diploids from heterokaryons				
1//2	S	6.0	45	7.3
1//3	S	6.3	41	ND
1//4	S	6.3	44	ND

ND = not determined.

*R = resistant and S = sensitive to the benlate test (Upshall *et al*., 1976).

Table III - Number of parental and recombinant type segregants isolated from heterozygous diploids.

	leuA_1		*bwn*A_1		*gre*A_1		*pab*A_1		*arg*A_1		*arg*B_2		*ebr*A_1	
	P*	R**	P	R	P	R	P	R	P	R	P	R	P	R
morA_1	55	124	96	93	96	93	36	48	48	0	57	0	40	44
leuA_1			125	118	125	118	36	48	26	56	28	54	41	43
bwnA_1					***		43	41	40	38	44	37	84	0
greA_1							43	41	40	38	44	37	84	0
pabA_1									nd		nd		43	41
argA_1											nd		nd	
argB_2											_		nd	

* parental type segregants; ** recombinant type segregants; *** epistatic genes; nd, crosses not done.

It has been observed that the *mor*A_1 marker is not easily detected, especially when it is together with the *leu*A_1 gene, because of the pleiotropic effect of the latter on morphology. In some cases it was not possible to distinguish between *mor*A_1 and *mor*A^+ strains and these sectors were not considered for analysis.

Two strains carrying markers on each linkage group detected, as well as the former strains are available upon request as we are interested in establishing a reference strain for genetical studies with *A. awamori*.

Interspecific crosses

Several attempts were made using Roper's technique (1952) and in no case were heterokaryons or interspecies hybrids detected, even when a different strain of *A. niger* was used (data not shown). In view of this protoplast fusion techniques were tried using *A. awamori pro*A_1 *leu*A_1 *bwn*A_1 and *A. niger pab*A_1 *nic*A_1 *olv*A_3 strains. After fusion treatment only 0.7% of the protoplasts that regenerated on CM plus 0.6 M KCl showed *A. niger* morphology which corresponds to 1×10^{-3} of survival. On the other hand 15% of *A. awamori* protoplasts survived, which suggests that the former species is much more sensitive to fusion treatment. Nevertheless 113 very small sterile colonies appeared on MM plus 0.6 M KCl out of 2.9×10^4 colony forming units estimated on CM plus KCl. This corresponds to a 0.3% fusion frequency. This figure is lower than some reports in the literature for interspecies crosses (Kevei and Peberdy, 1977) and is comparable to that obtained with *A. awamori* var. *kawachi* and *A. oryzae* fusion experiments (Ogawa *et al.*, 1988b). One of the causes for such a low frequency may be the higher sensitivity of *A. niger* protoplasts to fusion treatment. An alternative, which is now being considered, is electrofusion, as several other crosses are being made between these two species, aiming to study complementation of genes blocking glucoamylase production in *A. niger* (Bonatelli Jr. *et al*., 1984; Oliveira and Bonatelli Jr., data not published).

These putative fusion products were incubated up to 15 days though they maintained the same size and morphology observed three days after plating. Eleven of these were transfered to fresh MM and CM (half of the colony to each medium). On CM there was a vigorous growth and abundant conidiation in every case and colonies showed *A. awamori* and mixed growth morphology. On MM growth was very poor with very few conidiophores, if any. Subsequent transfers to fresh medium gave the same results in every case. This situation is similar to that reported with *A. nidulans* and *A. rugulosus* fusion experiments (Kevei and Peberdy, 1977). Conidia taken from mixed growth morphology on CM yielded almost exclusively (98%) *A. awamori* colonies when plated on CM and 1.1 x 10^{-5} mixed growth colonies when plated on MM. To ensure a strong selective pressure, putative fusion products were maintained on MM and occasionally small pieces of mycelium were transfered to CM. After 3-4 sub-cultures on MM one of the putative fusion products yielded several black conidial sectors when transfered to CM and MM. These sectors were quite vigorous and heavily conidiated on both media, indicating complementation of conidial color and auxotrophy by wild type genes. After two steps of purification, one colony was identified as an interspecies hybrid (Table IV).

Table IV - Response to benlate, conidial diameter, DNA content per nucleus and percentage of multinucleated conidia of *A. awamori*, *A. niger* and interspecies hybrid.

	Response to benlate (1.5 μg/ml)*	Conidial diameter (μm)	DNA/nucleus (μg . 10^{-8})	Percentage of multinucleated conidia
Strains				
proA_1 *leu*A_1 *bwn*A_1	R	4.9	3.6	45
pabA_1 *nic*A_1 *olv*A_3	R	4.8	3.1	52
Interspecies hybrid	S	6.0	7.0	48

*See Table II.

That colony behaved as an intraspecies diploid in all parameters studied (Table III; Bonatelli Jr. *et al*., 1983). It also showed spontaneous segregation on CM, and upon growth on benlate-containing CM, brown and olive segregants were easily detected and isolated. Interestingly this interspecies hybrid showed an starch index almost half (1.2) of that observed in *A. awamori* strains (1.5), indicating semidominance. That was not the case when glucoamylase was assayed on fermentation medium (Bonatelli Jr. *et al*., 1984) as the yield was similar to that observed for *A. niger* strains (Oliveira and Bonatelli Jr., data not published). These results suggest that interaction between genes involved in glucoamylase production determines a predominance of *A. niger* factors.

When segregants were isolated after benlate induction, it was observed that all of them were haploid and none showed the *leu*A_1 phenotype out of the 47 that were studied. When these had their starch index estimated 6% had an increased starch index (> 1.5) compared to the *A. awamori* parental strain. However these segregants had a glucoamylase yield similar to the interspecies hybrid, which probably indicates that there is no close relationship between the halo on starch-containing medium and the actual glucoamylase yield (Vialta, 1987). This is also apparent when *A. niger* strains are considered because they do not show a halo and they produce a reasonable amount of glucoamylase (Bonatelli Jr. *et al.*, 1984).

Auxotrophic and morphological characteristics of the segregants were also determined (Table V). Linkage relationships determined with intraspecies diploids in *A. niger* were also observed, e.g., there is linkage between *nic*A_1 and *olv*A_3 and *pab*A_1 is located in another linkage group (Bonatelli Jr. *et al.*, 1983; Masiero and Bonatelli Jr., 1989). Just one result is comparable to the *A. awamori* intraspecies diploid analysis, e.g., there is no association between *bwn*A_1 and *leu*A_1 (Table III). These results suggest that linkage relationships are preserved even in the new combination as has been observed with *A. nidulans* and *A. rugulosus* interspecific hybrids (Bradshaw *et al.*, 1983). However some data suggest that new linkage relationships could be detected with interspecies segregants. The clearest relationship was between *A. awamori* *bwn*A_1 and *shh*A_1 markers, that could not be linked in intraspecies crosses because all strains derived from NRRL 3112 form a starch hydrolysis halo (*shh*A_1). That association was also detected when the *A. niger* *nic*A_1 *olv*A_3 linkage group was considered, suggesting that the linkage group I of *A. awamori* has a certain homology to that of the former species (Masiero and Bonatelli Jr., 1989). If these linkage relationships are considered recombinant type segregants that appeared in that sample, they should be attributed to mitotic crossing over as they behaved as true haploids by all criteria employed in this work. Therefore, there would be enough homology at the chromosomal level to allow mitotic crossing over to occur. Homology between *A. niger* and *A. awamori* has been detected when the glucoamylase gene and flanking regions were compared at the nucleotide level and showed to be almost identical (Boel *et al.*, 1984; Nunberg *et al.*, 1984). Two segregants with complementary genetic markers were chosen and heterokaryons between them were obtained by Roper's technique (1952). One of them beared markers complementary to the *A. awamori* parental strain and the other to the *A. niger* parental strain. When combined with parental strains, heterokaryons were only detected as vigorous sectors for *A. awamori* and the segregant complementary to it. This may indicate that genetic factor(s) responsible for incompatibility between these two species can recombine, indicating that the barrier to hybridization between these two species could be allocated to linkage groups if crosses to strains bearing one genetic marker on each linkage group were made.

Table V - Number of parental and recombinant type segregants from interspecies hybrid.

		A. niger						*A. awamori*					
		*nic*A1		*pab*A1		*olv*A3		*bwn*A1		*leu*A1		*shh*A1	
		P	R	P	R	P	R	P	R	P	R	P	R
	*pro*A1	37	10	21	26	38	9	38	9	33	14	33	14
	*bwn*A1	46	1	26	21		*			28	19	42	5
A. awamori	*leu*A1	27	20	19	28	28	19					23	24
	*shh*A1	43	4	27	20	42	5						
A. niger	*nic*A1			27	20	46	1						
	*pab*A1					26	21						

* Not considered, possibly epistatic markers.

Taking all these results together it seems reasonable to suggest that these two species show a strong barrier to hybridization at the hyphal wall, and its removal through protoplast formation allows a more extensive interaction at the cytoplasmic level. The latter seemed to be less restrictive than the former because nuclear fusion was found to occur with the isolation of an interspecies hybrid after a protoplast fusion showed characteristics and behaviour similar to intraspecies diploids. Although segregation analysis was done in a relatively small sample of segregants and with few genetic markers, evidence of a relatively free linkage group reassortment has been obtained and linkage between *A. awamori* markers could be detected as well as a possible equivalence of one linkage group on both species.

Certainly studies that are now in progress will contribute to a more extensive knowledge about genetic interactions and also could provide more evidence about the possible close phylogenetical relationship between these two species as indicated by taxonomical studies (Raper and Fennell, 1965).

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RESUMO

Mutantes auxotróficos, morfológicos e resistente foram isolados de *Aspergillus awamori*, linhagem NRRL 3112, e usados para análise genética via ciclo parassexual. Resultados indicam um mínimo de quatro grupos de ligação para esta espécie. Híbridos interespecíficos com *Aspergillus niger* foram obtidos apenas após fusão de protoplastos e, a análise de segregantes sugere que: I) análise genética pode ser feita pois há confirmação de grupos de ligação detectados por diplóides intraespecíficos; II) associação no mesmo grupo de ligação de marcas de *A. awamori* e, III) homologia de grupos de ligação entre as duas espécies que permitiu a ocorrência de permuta mitótica. Estas sugestões foram consideradas como indicações da proximidade filogenética entre estas espécies.

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