Comparative sequence analysis of citrate synthase and 18S ribosomal DNA from a wild and mutant strains of *Aspergillus niger* with various fungi

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Abstract:
A mutation was induced in *Aspergillus niger* wild strain using ethidium bromide resulting in enhanced expression of citric acid by three folds and 112.42 mg/mL citric acid was produced under optimum conditions with 121.84 mg/mL of sugar utilization. Dendograms of 18S rDNA and citrate synthase from different fungi including sample strains were made to assess homology among different fungi and to study the correlation of citrate synthase gene with evolution of fungi. Subsequent comparative sequence analysis revealed strangeness between the citrate synthase and 18S rDNA phylogenetic trees. Furthermore, the citrate synthase movement suggests that the use of traditional marker molecule of 18S rDNA gives misleading information about the evolution of citrate synthase in different fungi as it has shown that citrate synthase gene transferred independently among different fungi having no evolutionary relationships. Random amplified polymorphic DNA (RAPD-PCR) analysis was also employed to study genetic variation between wild and mutant strains of *A. niger* and only 71.43% similarity was found between both the genomes. Keeping in view the importance of citric acid as a necessary constituent of various food preparations, synthetic biodegradable detergents and pharmaceuticals the enhanced production of citric acid by mutant derivative might provide significant boost in commercial scale viability of this useful product.

Abbreviations: CS, Citrate synthase; CA, Citric acid; RAPD, Random amplified polymorphic DNA; TAF, Total amplified fragments; PAF, Polymorphic amplified fragments; CAF, Common amplified fragments.

Background:
The condensation of acetyl-CoA with oxaloacetate is catalyzed by citrate synthase (CS, E.C. 4.1.3.7) to form citrate and coenzyme A. CS is involved in the well-established metabolic pathway of citric acid biosynthesis [1]. Although a number of microorganisms have been reported for citric acid production, *Aspergillus niger* is found to be the principal industrial producer of citric acid [2]. With many applications, citric acid (CA) is considered as a specialty chemical and is used in food and beverages, cosmetics, pharmaceutical, biomedicine and agriculture [3]. Among others, the advance applications of citric acid are also coming to light such as in biopolymers, fermentation of scaffolds for culturing wide variety of cell lines, drug delivery agents, bioremediation, wood preservation,

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removal of post soldering flux residues and in hydrometallurgy [3]. In 2004 the global production of citric acid was about 1.4 million tons [4] and the global market for citric acid is expanding every year with more than 5% per year.

Keeping in view the importance of citric acid, and more importantly *A. niger* as citric acid producer, we mutated and overexpressed the gene encoding citrate synthase and determined the increased activity of citrate synthase on citric acid production. In this study the phylogenetic tree constructed from citrate synthase fragment was compared to the tree derived from a traditional marker molecule to study how citrate synthase gene was transferred among different fungi. Independent evolution of citrate synthase gene would be suggested by the dissimilar phylogenies. To represent the evolution of fungi, the smaller ribosomal subunit (18S ribosomal DNA [rDNA]) was chosen. Of its nature, this is for the first time that we compared phylogenetic analysis of citrate synthase and partial 18S rRNA gene sequences from 30 different fungi including wild and our mutant of *A. niger*.

We set out to extend our analysis of citrate synthase fragments to measure polymorphism between wild and our previously mutated strains of *A. niger*. RAPD-PCR was employed which is a simple and convenient technique for DNA fingerprinting and genetic mapping [5]. This would open up the possibility of using the results for many applications while combinatorial biosynthesis is the most significant one.

Methodology:
Chemicals
The chemicals were purchased from Sigma Chemical Co., Missouri USA, unless otherwise stated.

Fungal strain and Mutagenesis using Ethidium bromide
Aspergillus niger strain ANJ-120 was obtained from Department of Microbiology, University of Agriculture, Faisalabad, Pakistan and subjected to mutagenesis using Ethidium bromide as we described previously [6]. 2-deoxy, D-glucose as selective marker was used to select the mutants and citric acid producing yellow brown colored colonies were selected for further studies. Mutants were checked for citrate synthase production and best producer was further used. Later, the best mutant strain of *A. niger* was named as EB-3.

Culture conditions and DNA template preparation
Aspergillus niger strains ANJ-120 and EB-3 were maintained at 4 oC after growing for 7 days in MYG medium (0.2% malt extract, 0.2% yeast extract, 2% glucose and 2% agar) at 28 oC [7]. The fungus was cultivated in Vogel's medium for 5 days at 28 oC with shaking at 120 rpm [8] with 1% glucose as a carbon source. For DNA isolation, both wild and mutant strains of *A. niger* were grown in Vogel's medium [9] supplemented with 2% glucose. DNA was extracted as described previously [10] with some modifications.

PCR amplification of 18S rDNA and citrate synthase gene fragments
The 18S rDNA was amplified from wild and mutant strains of *A. niger* with primers 5'-TCC GTA GGT GAA CCT GCG (sense primer) and 5'-CAT ATG CTT AAG TTG AGC GGG (antisense primer). To isolate a partial DNA encoding citrate synthase

from both strains using PCR, oligonucleotides were designed and the sequences of the primers were 5'-TCT TTC TCT CCT CTC TTT CTT TCC TTC CAA CCA C (sense primer) and 5'-CAG AAG ACG ATT GAC TTG GAA TTT CTC AAG TGC TGT T (antisense primer).

Taq DNA polymerase with 10X PCR buffer, MgCl2 and dNTPs were obtained from Fermentas. The amplification of 18S rDNA was carried out under the following conditions: 30 cycles of 1 min at 94 oC, 1 min at 42 oC, and 1 min at 72 oC, with a final extension of 10 min at 72 oC. While the amplification of citrate synthase was carried out under the conditions of 30 cycles of 1 min at 94 oC, 1 min at 55 oC, and 1 min at 72 oC, with a final extension of 15 min at 72 oC.

Cloning and sequencing of the amplified PCR fragments
The amplicons of expected sizes for 18S rDNA and citrate synthase were gel purified with a Qiagen QIAquick PCR cleanup kit using protocols provided by the manufacturer. The fragments were cloned and sequenced with the same sets of primers from the Centre for Applied Molecular Biology (CAMB), Lahore, Pakistan.

Phylogenetic Analysis
The forward and reverse sequences of both 18S rRNA and citrate synthase genes sequenced in this study were assembled and analyzed using BLAST (Basic Local Alignment Search Tool) [11] available on the NCBI website (http://www.ncbi.nlm.nih.gov/). Along with our wild and mutant sequences for 18S rRNA and citrate synthase genes of *A. niger* twenty eight most similar sequences of different fungi were also retrieved from GenBank for the comparison study. All the sequences were aligned using ClustalX and imported into the Bioedit program [12] for manual alignment. Neighbor-joining phylogenetic trees were constructed for 18S RNA and citrate synthase genes using the program MEGA5 [13] with 100 bootstrap replicates.

RAPD-PCR analysis
DNA fingerprinting was performed with a set of fifteen 10-base oligonucleotide RAPD primers (OPA-1, OPA-4, OPA-5, OPA-7, OPA-9, OPC-4, OPC-5, OPC-6, OPC-8, OPC-10, OPC-11, OPC-14, OPE-2, OPE-3, OPE-6) obtained from Operon Tchnologies, Inc., Aalamada, CA, USA. A total of 25 µL reaction volume was made for PCR amplification, containing 3 mM MgCl2, 0.1 mM each of dNTPs, 30 ng of primer, 0.001% gelatin, 25 ng of DNA and 1 unit of Taq DNA polymerase. Amplifications were carried out in Eppendorf Mastercycler Gradient, programmed for a first denaturation step of 5 min at 94 oC followed by 40 cycles of 94 oC for 1 min, 36 oC for 1 min, 72 oC for 2 min and one cycle at 72 oC for 10 min, and then held at 20 oC until the PCR tubes were removed [14]. The products were electrophoresed on 1.2% agarose gel using 0.5X TBE buffer and visualized under UV light after staining with ethidium bromide and photographed on EagleEye Gel Documentation System. RAPD amplification was repeated two times and reproducible, explicitly score-able fragments were considered only. For the analysis of genetic diversity, all the polymorphic loci were scored as present/absent. To estimate the similarity between both genomes on the basis of number of shared amplification products, the bi-variate 1-0 data was used as done by [15].
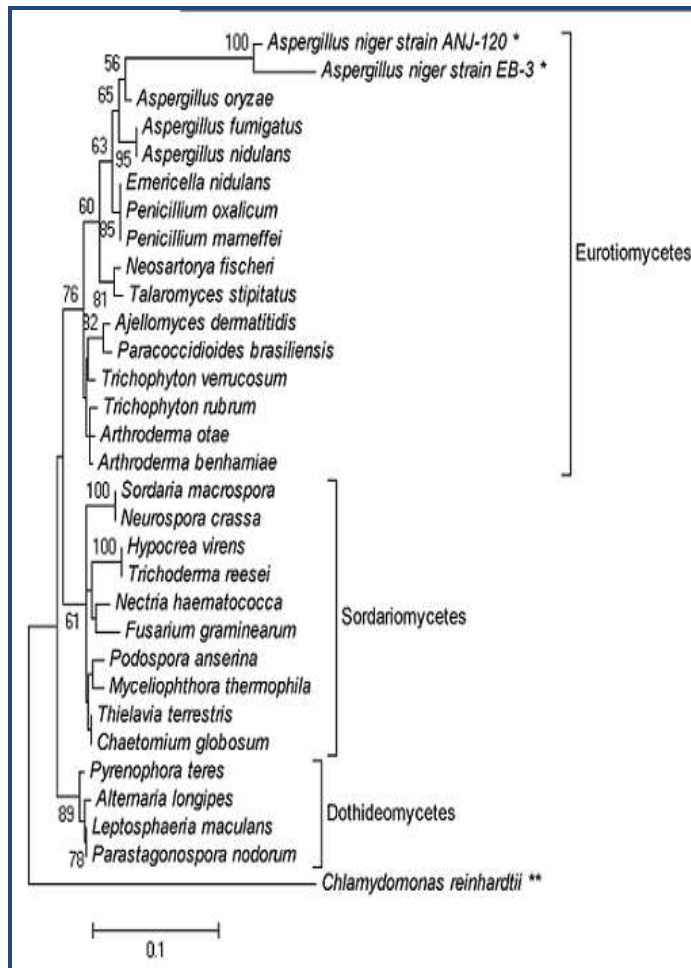
Figure 1: Phylogenetic tree constructed from partial 18S rDNA sequences. Bootstrap values greater than 55% are shown at the nodes. Sequences marked with asterisks are the sample organisms and the sequence with double asterisks is used to root the tree. The nucleotide sequences accession numbers are as follows: *Ajellomyces dermatitidis*, XM_002625349; *Hypocrea virens*, AF501334; *Pyrenophora teres*, AF400892; *Podospora anserina*, GU391422; *Nectria haematococca*, AJ131323; *Trichophyton rubrum*, HQ537428; *Aspergillus oryzae*, JN227035; *Neosartorya fischeri*, JN943575; *Penicillium oxalicum*, JN851007; *Aspergillus fumigatus*, JN851039; *Emericella nidulans*, JN638778; *Paracoccidioides brasiliensis*, JQ675762; *Penicillium marneffei*, JQ912276; *Talaromyces stipitatus*, JX122729; *Leptosphaeria maculans*, JX648199; *Myceliophthora thermophila*, JX868606; *Sordaria macrospora*, KC171332; *Trichophyton verrucosum*, KC833522; *Fusarium graminearum*, KC810065; *Neurospora crassa*, JX981479; *Thielavia terrestris*, KF313105; *Arthroderma otae*, KC923429; *Parastagonospora nodorum*, KF512822; *Chaetomium globosum*, HG530326; *Trichoderma reesei*, KF294851; *Arthroderma benhamiae*, KF437401; *Aspergillus nidulans*, KC473931; *Alternaria longipes*, KF573971; *Aspergillus niger* strain ANJ-120, JN587346; *Aspergillus niger* strain EB-3, KF997092; *Chlamydomonas reinhardtii*, KF997092.

Results:
After treating for 120 min with ethidium bromide the EB-3 strain of *A. niger* was selected as best mutant as it showed best growth in the presence of selective marker. The colonies that were turned to yellow brown color were selected. For further selection on the basis of maximum citric acid production from these petri plates five colonies were picked and grown on slants.

Analysis of the 18S rDNA tree
The gene encoding 18S rDNA subunit was amplified from wild and mutant strains of *A. niger* and analyzed using BLAST. The partial nucleotide sequences corresponding to the gene encoding 18S rRNA were deposited in the GenBank and allotted accession numbers JN587346 and KF997092 for ANJ-120 (wild) and EB-3 (mutant) strains of *A. niger* respectively. In addition to our wild and mutant strains of *A. niger* which belongs to Eurotiomycetes class, 28 closely related sequences of different fungi from three classes i.e. 14 sequences from Eurotiomycetes, 10 sequences from Sordariomycetes and 4 sequences from the class Dothideomycetes were added to the analysis. All these sequences were used to construct a multiple alignment and a phylogenetic tree, which is shown in (**Figure 1**). *Chlamydomonas reinhardtii* 18S rRNA sequence was used as an outgroup in the analysis. The evolutionary history was inferred using the Neighbor-Joining method [16]. The tree is fully in accordance with the fungal classification system and all the fungi fall in their respective classes. By using only partial 18S rDNA it was revealed by the bootstrap analysis of the tree (100 replicates) that all of the phylogenetic relationships were not fully resolved. However the clearly separable clades in the tree can be used to compare the fungi found in these branches to the fungi in the citrate synthase tree to investigate the similarity of the two trees.
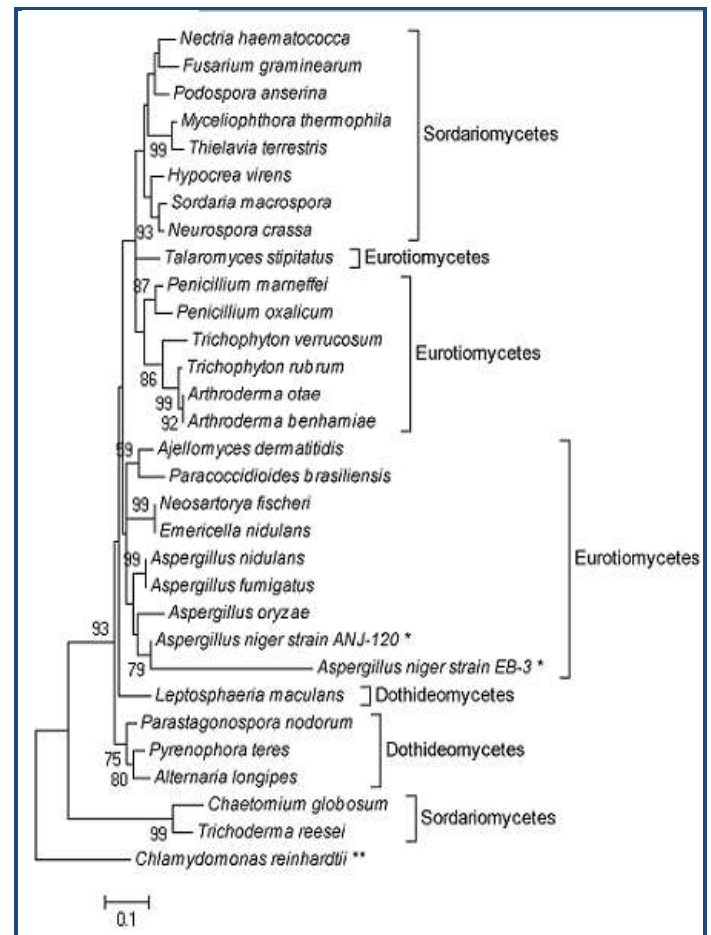

Figure 2: Phylogenetic tree constructed from CS gene fragments. Bootstrap values greater than 55% are shown at the

nodes. Sequences marked with asterisks are the sample organisms and the sequence with double asterisks is used to root the tree. The nucleotide sequences accession numbers are as follows: *Ajellomyces dermatitidis*, XM_002623627; *Hypocrea virens*, DQ456855; *Pyrenophora teres*, XM_003303988; *Podospora anserina*, XM_001909446; *Nectria haematococca*, XM_003049642; *Trichophyton rubrum*, XM_003235026; *Aspergillus oryzae*, XM_001822722; *Neosartorya fischeri*, XM_001266109; *Penicillium oxalicum*, GQ981487; *Aspergillus fumigatus*, XM_742827; *Emericella nidulans*, AF468824; *Paracoccidioides brasiliensis*, XM_002789962; *Penicillium marneffei*, XM_002149057; *Talaromyces stipitatus*, XM_002485033; *Leptosphaeria maculans*, XM_003840428; *Myceliophthora thermophila*, XM_003667071; *Sordaria macrospora*, XM_003350683; *Trichophyton verrucosum*, XM_003021136; *Fusarium graminearum*, XM_381598; *Neurospora crassa*, XM_951805; *Thielavia terrestris*, XM_003657993; *Arthroderma otae*, XM_002844827; *Parastagonospora nodorum*, XM_001792322; *Chaetomium globosum*, XM_001222818; *Trichoderma reesei*, DQ849026; *Arthroderma benhamiae*, XM_003014468; *Aspergillus nidulans*, XM_676452; *Alternaria longipes*, HQ171096; *Aspergillus niger* strain ANJ-120, KC847093; *Aspergillus niger* strain EB-3, JF415912; *Chlamydomonas reinhardtii*, XM_001702931

Analysis of the citrate synthase (CS) tree

The partial nucleotide sequences of citrate synthase gene from wild and mutant strains of *A. niger* were also deposited to GenBank which appeared under accession numbers KC847093 and JF415912 respectively. A Neighbor-Joining tree was constructed from the nucleotide sequences encoding citrate synthase gene of the same 30 fungi of Eurotiomycetes, Sordariomycetes and Dothideomycetes classes which were used to construct 18S rDNA tree. The distantly related citrate synthase gene from *Chlamydomonas reinhardtii* was used as an outgroup **(Figure 2)**. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches [17]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [18] taking transitions and transversions into account and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons [19]. All positions containing gaps and missing data were eliminated.

Incongruities between the two phylogenetic trees

Comparison of the distribution of selected fungi in two phylogenetic trees shows that there seems to be no correlation between CS and 18S rDNA sequences. For instance, in 18S rDNA tree Eurotiomycetes contains all the 16 fungi of this class in one major clade but in CS tree they have been divided into three separate subclades. *Talaromyces stipitatus* does not fall in any clade of its class and making a separate one. Like 18S rDNA all the four species of *A. niger* are closely related in CS tree as well and the bootstrap value of 79% is justifying the closeness of wild and mutant strains of *A. niger* Similarly, *Pyrenophora teres, Alternaria longipes, Leptosphaeria maculans* and *Parastagonospora nodorum* were selected from the class Dothideomycetes which appear in one clade in 18S rDNA but in CS tree *Leptosphaeria maculans* gets separated from the other

three fungi. The most interesting case is with the class Sordariomycetes. The class contains 10 fungi in total which appear in one major clade in 18S rDNA tree but in CS tree they are behaving differently and 8 of them are falling in one clade at the top of the tree while *Chaetomium globosum* and *Trichoderma reesei* are making a separate clade at the base of the tree and are separated by a great phylogenetic distance. In addition to above described examples numerous other discrepancies can also be found in two phylogenetic trees. Taken together, the results of phylogenetic studies strongly imply that the evolution of citrate synthase is independent of the evolution of fungal species. Finally, it should be noted that the 18S rDNA and CS tree comparisons were made by using trees which contain sequences appearing in both trees.

Citric acid production by A. niger transformant overproducing citrate synthase

When we compared the activity of citrate synthase gene from the mutant strain of *A. niger* with the wild one, it was observed that citric acid production by the mutant EB-3 was significantly enhanced i.e. from 19.4 to 64.20 mg/mL which was further enhanced to 112 mg/mL [20] by optimizing fermentation parameters and by adding suitable surfactant, nitrogen source, metabolic inhibitor and metal ion complexing reagent (data not shown). Sequence comparison of citrate synthase from both strains revealed that in addition to substitutions, deletion mutations in the mutant EB-3 strain of *A. niger* were observed that resulted in hyper-expression of citrate synthase.

DNA fingerprinting

In this study, we delivered and compared the genomes of two strains of *A. niger* i.e. ANJ-120 and EB-3. Both strains appeared having some different phenotypes: the EB-3 predecessor to efficient citric acid producing strain having undergone some level of mutagenesis, and the other ANJ-120, a wild-type parent strain. This finding makes the comparison interesting both in terms of genomic research and industrial applications of citric acid. In total 31 primers were used to amplify the genomic DNA in both the strains that gave sufficient polymorphism between the two *A. niger* isolates. A total of 98 score-able amplified DNA fragments ranging in size from 250 bp to 3.5 kb base pairs were observed using 15 primers of the three series **Table 1 (see supplementary material)**, with an average of ~6 fragments per primer. It was observed that 28 fragments were polymorphic (PAF) and 70 bands were monomorphic (CAF) between isolates of A. niger. The fifteen primers showed polymorphic percentage 28.57%, as well as among amplified 70 monomorphic bands with percentage 71.43%. Results of this effort showed that there is a significant change between the genetic makeup of *A. niger* ANJ-120 and *A. niger* EB-3.

Discussion:

The improvement of citric acid producing strains by mutagenesis and selection is not a new field. Use of different mutagens to induce mutations in the parental strains has been the most employed technique [4]. Ultra violet radiations, γ-radiations and chemical mutagens are mostly used to induce mutations. For commercial production mutants of *A. niger* are used [21] and UV treatment combined with some chemical mutagens is mostly used to get hyper-producer strains of *A. niger*. Enhanced production of citric acid by mutagenesis of *A. niger* has been reported by some workers. For example,

mutations introduced by ultraviolet-irradiation, ethyl methane sulfonate and acridine orange enhanced the citric acid production to 3.2 fold in *A. niger* [22]. Gamma-rays irradiation on *A. niger* mutants also improved the production of citric acid significantly [23].

Recent studies of genetic engineering tools [24] have revealed that the filamentous fungi have become an important source of certain compounds. These are gifted with high yields and secretion capacity in addition to favorable media requirements [25]. *Aspergillus niger* is an important producer of citric acid on industrial scale because its fermentation processes have been well studied and tested, and the fungus has earned a status of 'generally regarded as safe' which has made it an attractive host for biotechnological production [26]. For citric acid production, citrate synthase is a key enzyme and its activity is observed throughout the acidogenic growth of *A. niger* [27]. Although the sequences of citrate synthase from different fungi are known but prior to its genome publication [28], the *citA* gene was studied from only two strains of *A. niger* although their focus was on the production of citric acid and not on the promoter of *citA* [29].

The phylogenetic analysis showed that *A. niger* ANJ-120 and *A. niger* EB-3 clustered together in both trees. The study of gene and genome evolution has been revolutionized by the introduction of molecular techniques and allowed new approaches to phylogenetic inference. In particular, when biochemical features have proven to be insufficient then molecular sequences are useful for comparisons among distantly related species [30]. For phylogenetic studies NJ method has been reported to be a better alternative to maximum parsimony method [31]. NJ and maximum likelihood methods have been shown to be generally more efficient and nearly equally efficient than the maximum parsimony method [32]. A high variability was also observed between genome sequences of *A. niger* ATCC 1015 and *A. niger* CBS 513.88 through phylogenetic analysis [33].

In this study we collected 30 sequences of different fungi along with our wild and mutant strains of *A. niger* for traditional 18S rDNA and citrate synthase gene because to gain insight into the evolutionary relationships of different fungi 18S rDNA has been extensively used [34]. The data presented here shows that there is no evident correlation between the phylogenetic trees constructed by using these different molecules. The results also show that the fungi which are clustered together in 18S rDNA phylogenetic tree fall in separate clades when tried for citrate synthase phylogenetic tree and found distantly related. These results strongly suggest that citrate synthase gene transferred independently among different fungi having no evolutionary relationships. It is significant that there are no inconsistencies in the CS tree and the fungi of the same class are not represented in the same clade of the tree.

To study genetic variation between species RAPD-PCR is a powerful tool but reproducibility is the major concern in the amplification of RAPD markers [35]. To differentiate different strains of *A. niger* and other strains random primers have been successfully employed worldwide [15]. In those developing countries where advanced facilities are not available, RAPD-PCR technique can be utilized effectively to differentiate

between wild and mutant cells, intra-specific and inter-specific strains of different organisms [15]. We emphasized RAPD-PCR to verify whether the wild strain of *A. niger* was genetically different from the mutant one or not. To overcome the reproducibility problem, in the present study only major/bright DNA fragments were scored. Because of random priming nature of PCR reaction the minor fragments were not considered [36].

In conclusion, we successfully subjected *Aspergillus niger* ANJ-120 to mutagenesis by treating with ethidium bromide for hyperproduction of citric acid. Citrate synthase gene of wild and mutant was amplified, sequenced and compared. RAPD-PCR was performed which confirmed a momentous change in the genetic makeup of mutant strain. Only 71.43% similarity was found between both the genomes which suggested that there is a potential difference between these two strains of A. niger. Mutant strain of *A. niger* could easily be differentiated from the wild strain by its unique amplification products which are the results of acquired genetic variation by mutation. No correlation was observed between 18S rDNA used for traditional evolutionary study of fungi and citrate synthase phylogenetic trees. Enhanced production of citric acid from the mutant strain might provide a very significant boost to muddle through the economic expediency and commercial scale feasibility of this useful product.

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Supplementary material:

Table 1: The three series of random primers that produced polymorphic bands useful for observing genetic variability between two strains of *Aspergillus niger*

Series of random primers	Number of random primers applied	TAF	Number of PAF	%PAF	Number of CAF	%CAF
OPA	5	37	11	29.7	26	70.3
OPC	7	36	10	27.8	26	72.2
OPE	3	25	7	28	18	72
Total	15	98	28	28.57	70	71.43