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Molecular characterization of six tannase-producers *Aspergillus* strains using PCR-RFLP of ITS and IGS regions and RAPD´s

V.Padilla-García[1], F.Castillo-Reyes[4], C.N.Aguilar-González[1], A.Cuenca-Arana[2], A.Téllez-Jurado[2], M.H.Reyes-Valdez[3], R.Rodríguez-Herrera[1]*

[1]Department of Food Research, School of Chemistry, Universidad Autónoma de Coahuila, Blvd. V. Carranza and José Cárdenas V. s/n. Col. RepublicaOte. Zip code 25280, Saltillo, Coahuila, (MÉXICO)

[2]Biotechnology Department Universidad Autónoma Metropolitana- Iztapalapa, (MÉXICO)

[3]PlantBreeding Department, Universidad Autónoma Agraria Antonio Narro, Buenavista, 25315 Saltillo, Coahuila, (MÉXICO)

[4]Saltillo Experimental Station, INIFAP. Carretera Saltillo-Zacatecas, km 342+119. Núm. 9515, Colonia Hacienda de Buenavista, Saltillo, Coahuila, (MÉXICO)

E-mail : rrh961@hotmail.com

ABSTRACT

This study aimed to: determine the genetic differences among six tannase-producing fungal strains using three molecular techniques (RAPD's, IGS and ITS), establishing genetic relationships at the molecular level among the tested fungal strains of *Aspergillus* genus and compare the efficiency of molecular markers (ITS, IGS and RAPD's) to establish genetic relationships among fungal strains under study. Fungal species were isolated from plant tissue samples collected near Saltillo Coahuila Mexico, fungal strains were isolated from tissue plants such as *Pinuscembroides* and *Larreatridentata* (*Aspergillusniger* PSH and GH1 respectively) and soil near semidesert plants *Larreatridentata* (*Aspergillusfumigatus* GS), *Quercus*sp (*Aspergillusornatus* ESH), *Pinuscembroides* (*Aspergillusterricola* PSS) and as control was used *Aspergillusniger* (AA20) isolated from *Coffeaarabica* in the State of Veracruz. Visualization of amplified products was done using agarose gel (1.5%) electrophoresis, using ITS4, ITS5, IGSR IGSF primers it was possible to amplify by PCR fragments with size from 600 to 800 bp, revealed amplification of the 18 S rDNA. The results showed that it is possible to identify highly related fungal strains using PCR-RFLPs of IST and IGS regions. These results suggest that the nucleotide sequence of the ITS and IGS fragments is different. © 2014 Trade Science Inc. - INDIA

KEYWORDS

Aspergillusniger;
Aspergillusornatus;
Aspergillus fumigates;
Aspergillusterricola.

INTRODUCTION

Tannase enzyme is used extensively in the food industry, beverage, beer, pharmaceutical and chemical industries. This enzyme hydrolyzes the tannins molecule by its esterase activity[2]. Tannins are found in plants as protection components, so as in wastewater from tanneryand food industries. Tannins presence in waste-

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water difficult bioremediation with microorganisms, while in foods is significantly reduced quality because tannins confers taste bitter and harsh, astringent effect[12], also proteins precipitate and decrease sensitivity of taste. In the food industry, tannase is used for clarification of wines and juices. Moreover, hydrolysis of tannic acid producesgallic acid which has applications in the pharmaceutical industry[5]. Because various commercial applications that have this enzyme, there is a constant search for new sources of tannase with more desirable properties for industry. Some of the microorganisms reported degrading tannins are: *Aspergillus* spp. *Penicillium* spp. *Emericiella* spp. and *Candida* spp. The vast majority of these fungal species have been isolated from tropical regions[17] At the Autonomous University of Coahuila diverse fungal strains were isolated from the Coahuila State semi-desert[4]. These strains are important because they possess a greater tannase activity that some of the fungal strains isolated in tropical areas[4]. Furthermore, some of these strains have shown production of both intra and extracellulartannase[3]. Moreover, it is also reported that some of these strains do not only degrade hydrolysable tannins but also can degrade condensed tannins such ascatechin[1], based on the above needs to characterize the identity of these fungal strains, in order to identify if they are novel and therefore new sources of tannase enzyme. To identify these strainswe took advantage of the advances in molecular biology, which in the last three decades have provided genetic markers for viewing tangible differences among homologous DNA sequences. These differences result from changes or rearrangements between the base pairs such as translocations, inversions, additions or deletions in homologous regions[9]. DNA markers are obtained for different methods oneof them is based on the chain reaction polymerase (PCR) technique[8]. Some of the most used bookmarks in fungi are based on the variability that exists in some (ITS and IGS) regions of the ribosomal genes. The largest amount of variation in the rDNA sequence exists within the intergenic region space (IGS). The IGS region is a good candidate for strain differentiation at the intraspecific level[6]. The ribosomal genes are highly conserved and are linked internally by the ITS1 and ITS2 (internal transcribed spaces). Amplification of these regions, including the entire 5.8S gene has allowed molecular characterization to fungi at the genus, species and strain level[13]. Other markers used in the molecular characterization of organisms are RAPD's which is a technique for rapid screening of genomic polymorphisms using short primers of arbitrary sequence length to amplify by PCR specific areas randomly distributed throughout the genome[9]. This study aimed to: determine the genetic differences between six tannase-producing fungal strains using different molecular techniques (RAPD's, IGS and ITS), establishing genetic relationships at the molecular level among *Aspergillus* fungal strains and compare the efficiency of ITS molecular markers, IGS and RAPD's to establish genetic relationships among the fungal strains.

MATERIALS AND METHODS

Fungal strains

In this study we used the fungal strains isolated by Cruz[4], which were identified as *Aspergillusniger* (PSH and GH1) isolated from leaves of *Pinuscembroides* and *Larreatridentata* respectively, *Aspergillusornatus* (ESH) isolated from *Quercus* sp., *Aspergillusterricola* (PSS) and *Aspergillusfumigatus* (GS) isolated from soil where were grew *Pinuscembroides* and *Larreatridentata*, respectively. All samples were collected from the semi-desert near Saltillo Coahuila Mexico, and *Aspergillusniger* (AA20) isolated from *Coffeaarabica*at the Veracruz State. This fungal collection wasmaintained in the culture collection of the School of Chemical- the Autonomous University of Coahuila. Each fungal strain was actived in Potato Dextrose Agar medium (PDA) placing 2 µL of a spore suspension (7000-8000 sporesμL^{-1}) and incubated at 30 ° C for a period of 7 days.

DNA isolation

DNA extraction was performed using the technique reported by Raeder and Broda (1985), using mycelium of each strain which was growth in liquid medium Malta-based (20 g / L) at constant agitation. DNA was checked for integrity in agarose gels 1% and quantified spectrophotometrically the amount of DNA extracted. Afterthat PCR was perform to determine differences in the sequences of fungal in the regions ITS and IGS and RAPD's.

PCR Amplification of ITS and IGS regions

Amplifications of ITS and IGS regions were per-

formed using the following primers: ITS4 (5 'TCC TCC GCT TAT TGA TAT GC 3'), ITS 5 (5'GGA AGT AGT AAA AAG CGT AAC G 3 '), IGSF (5'CTG AAC TCT TCA GCC AAG G 3 ') and IGSR (5' AAT TTC CGA GAG GCA GTT CT 3 ') reported by White et al., (1990) and Edel et al., (1995) respectively. In anamplification total volume of 25 µL [2 µL DNA, 23 µL PCR mix composed by PCR buffer 1X (20nm Tris, pH 8.4, 50nMKCL) (Invitrogen), 0.2 mMdNTPs, 2.0 mM MgCl2, 0.5 uMprimer and 0.1 U / µL of Taq DNA polymerase (Invitrogen)]. PCR Amplification was performed in a thermocycler () under the following program: an initial cycle of 94 ° C for 4 min, followed by 35 cycles (94 ° C for 1 min, 50 ° C for ITS and 54 ° C for IGS for 1 min. and 72 ° C for 1 min, with a final elongation for 5 min at 72 ° C).

Digestion of ITS and IGS regions

The amplified DNA from ITS and IGS region was digested in an Eppendorf tube 0.6 µL, using the following reagents: 16.3 µL sterile distilled water, 1 µL amplified DNA, buffer 1X (250MM Tris-acetate pH 7.8, 1 M potassium acetate, 100 mM magnesium acetate and 10mm DTT), 1 µL acetylated bovine serum albumin, 0.25 $U\mu L^{-1}$ restriction enzyme. The enzymes used to digest IGS region were*: Cla I, Eco RI, Hind III* and *Pst I*,while ITS region was digested using *BamH, Hind* III, *Cla I* and *Xho I*. The amplified DNA (control) and restriction enzyme mix was incubated overnight at 37 ° C, and then samples were centrifuged briefly and were added 1 µl of bromophenol blue. The DNA digestion products were separated on polyacrylamide gels at 15% and stained with silver nitrate according to the procedures described by Kahl and Valadez[16].

Random amplified Polymorphic DNA (RAPD's)

For determination of RAPD markers were used three primers A02 (TGC GCT CGA G), A08 (TAG ACG GTG G) and D06 (TGA ACC ACG C), the PCR reaction was performed at a final volume of 25 µL [2 µ L DNA and 23 µL PCR mix composed by PCR buffer 1X (20nm Tris, pH 8.4, 50nMKCL) (Invitrogen), 0.2 mMdNTPs, 2.0 mM MgCl2, 0.5 uMprimer and 0.1 $U\mu L^{-1}$Taq DNA polymerase (Invitrogen)]. The thermocyclerprogram for RAPD's consisted in ainitial denaturation temperature of 94 ° C for 2 min, following by 44 cycles at 92 ° C for 1 min, 36 ° C for 1 min, 72 ° C for 2 min, and a final elongation at 72°C by 7 min. The samples were fractionated on an agarose (1%) gel.

Dendrograms

The results from ITS, IGS, RAPD's amplificationsand restriction pattern of IGS and ITS were coded as follows: present bands = 1, absent bands = 0 and lack of data = NA. Coded data were analyzed to perform dendograms using Phylipprogram[7], using modified Nei distances and the Neighbor-Joining method.

RESULTS AND DISCUSSION

Amplification ITS and IGS regions from fungal DNA

Once DNA was obtained, PCR was performto determine the differencesin the sequences from fungal ITS and IGS regions. Subsequently, amplified DNA productsof each sample were observed using an agarose (1%) gel. Figure1 shows the bands obtainedfrom fungal samples;A. *niger* (GH1), A. *niger* (PSH) and *A.niger* AA20, wherese gments of 700bp from the ITS region were amplified. In the case of PSS fungus a band of 600 bp was amplified. These results agree with those obtained by[10], whom detected *Phanerochaetechrysosporium* in soil by DNA amplification and subsequent restriction enzyme analysis, using the same primersemployed in this workand finding a 700 bp amplicon. In our study it was not possible to amplify the ITS region from ESH and GS strains (Figure 1), one possible explanation for these results is that

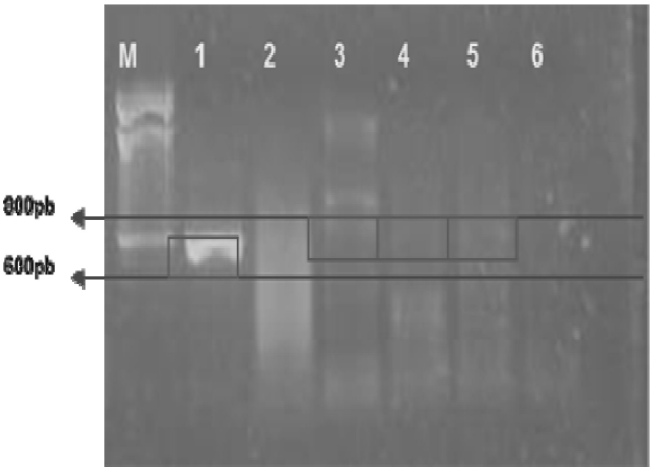

Figure 1 : PCR amplification of ITS region from tannase-producing fungal strains: M: molecular marker (Invitrogen DNA 100bp Ladder); 1=PSS; 2=ESH; 3=GH1; 4=AA20; 5=PSH; 6=GS.

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DNA contaminations or mutations within the amplified region prevented the primer coupling to the DNA strand, whereby no amplification occurred[16].

Figure 2 shows amplification of IGS region, where three segments (100, 400 and 700 bp) were amplified from PSS,GS and ESH fungal strains, four bands from PSH (100, 300, 400 and 900 bp), and GH1 strain (100, 300, 500 and 900 bp), and only two segments (100 and 400 bp) were amplified from AA20 control these being similar to those presented in PSS, ESH, GS and PSH; GH1fungal strains.

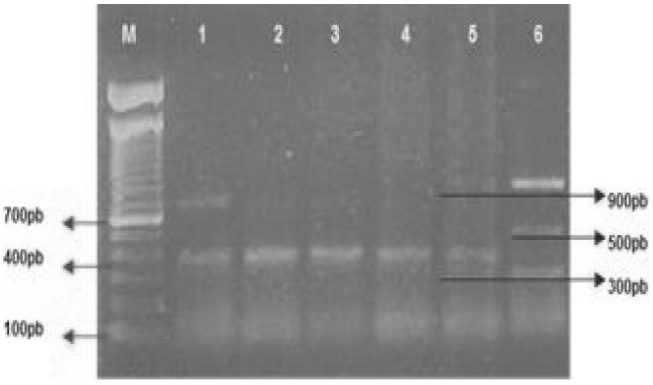

Figure 2 : Amplification of IGS region from different fungal strains: M= molecular marker (Invitrogen DNA 100bp ladder); 1=PSS; 2=ESH; 3=GS; 4=AA20; 5=PSH; 6=GH1.

The above results suggest that it is possible to identify these strains from each other despite being genetically related. It was also observed that PSS, GS ESH strains have a similarity size of the amplified fragments from the IGS region, despite being different species, it is important to check if nucleotide sequence of the fragments is different. The PSH, GH1 and control AA20 strains although are the same specie, showed differences in the nucleotide sequence in the amplified IGS region. One possible response to these differences is that these fungi were isolated from different hosts and regions indicating that diverged in evolution therefore had rearrangements at the DNA level.

Determination of RAPDs

The three primers for RAPD's (A02, A08 and D06) using in this investigation amplified fragments of size from 100 to 900 base pairs (Figures 3 and 4).

TABLE 1 shows a comparison of sizes of amplified bands by RAPD's from different fungal strains, it was observed that with the primer A02 was possible to detect a wide genetic variability among the fungal strains, since as shown in TABLE 1 all fungal strains presented

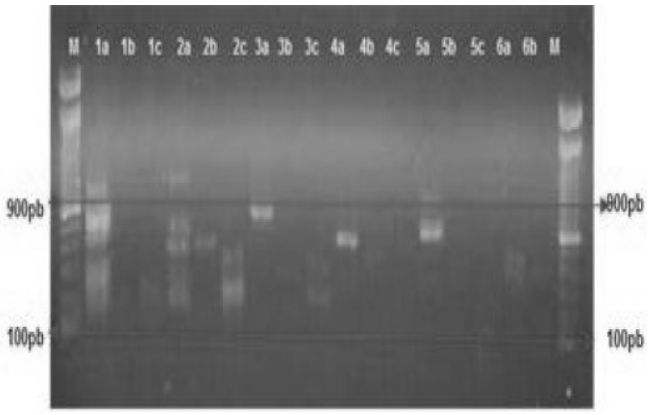

Figure 3 : DNA bands amplified from the tannase-producing fungal strains using the RAPD's primers (a) A02, (b) A08, (c) D06: M: (Invitrogen DNA 100bp ladder); 1a=PSS, 1b=PSS, 1c= PSS; 2a= ESH, 2b=ESH, 2c=ESH; 3a=GS, 3b=GS, 3c=GS; 4a=AA20, 4b=AA20, 4c=AA20; 5a=PSH, 5b= PSH, 5c= PSH; 6a= GH1, 6b= GH1, 6c= GH1.

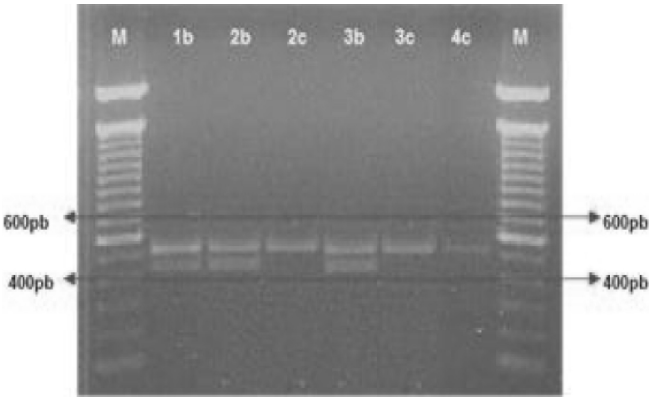

Figure 4 : DNA bands amplified from the tannase-producing fungal strains using the RAPD's primers (b) A08, (c) D06: M=Invitrogen DNA 100bp ladder; 1b=PSS; 2b=AA20, 2c=AA20; 3b=PSH, 3c= PSH; 4c=GH1.

different band patterns. In the PSS, ESH, GH1 and control AA20 strains was amplified a band of 500 bp. This fragment can be a non-specific region because it is

TABLE 1 : Comparison and differentiation of amplified segments (bp) with RAPD's of six strains of the genus *Aspergillus* producers of the tannase enzyme.

Fungalstrain	Primer A02	Primer A08	Primer D06
PSS	750, 500, 450, 300, 100.	550, 450	200
ESH	900, 500, 400, 200	450	400, 250
GS	700	300	350, 250
AA20 (control)	500	550, 450	550
PSH	900, 600	550, 450	550
GH1	500, 400	450	550

presented as equal in size from different fungus species.

The ESH strain presents a nonspecific band of 400 bp similar to that of the GH1 strain, the PSH strain presents a nonspecific band of 900 bp similar to that of the ESH strain, and the GS strain presented a band of 700 bp different from other fungal strains. The Amplified bands specific to a strain represent a potential source for the design of specie-specific primers. In general with the RAPD's study was observed, that all the strains used in this study can be differentiated with the primer A02, and by combining the results obtained with the primer A08 and D06 only 4 out of 6 (PSS, ESH, GS and GH1) strains can be identified, in contrast, the PSH, GH1 and AA20 strains exhibit similar polymorphism in almost all the RAPD's bands. The using of D06 primer permitted amplification of DNA fragments to identify *A. niger* from other fungi, therefore specific bands RAPD's can be used to design specie-specific primers, this methodology is known as SCAR (Region amplified characterized sequences)[8].

PCR-RFLP of ITS fragments

In order to find greaterdifferentiationamong the fungal strains, a DNA digestion study of the amplified ITS regionusing four restriction enzymes (*ClaI, Bam HI-Hind III*and*XhoI*) was conducted, and the different size fragment sare described in TABLE 2.

TABLE 2 : Restriction fragments (bp) obtained in DNA amplified from ITS region in four fungal strains

Key Fungi	Cla I	BamH - Hind III	Xho I
PSS	600, 300, 200	600, 520	600, 450, 220
PSH	600	700	600, 300
GH1	600	700	600, 300
AA20 (control)	600, 550	700	600

This study indicated that it is possible to identify tannase-producer fungal strainsby digesting ITS fragments using a combination of restriction enzymes, although in some fungi it was not possible to cut the amplified fragment. According[10] amplified product by PCR from *Phanerochaetechrysosporium* were cut with restriction enzymes; these authors observed restriction and amplified fragments with similar sizes that those observed in this study (bands from 600 to 800 bp), but is some fragments there were no restriction sites in the amplified fragments to the used enzymes. The best enzyme to identify the fungal strains in this study was *Xho I* which producedbands that allow discrimination among strains.

Digestion of IGS fragments byrestriction enzymes

In analyzingthe IGS fragments obtained after digestion with restriction enzyme cut from the 6strains, it was observed a similar digestion pattern from the IGS fragments with DNA from GH1 and GSstrains (TABLE 3).

The digestion of amplified IGS fragments with *Cla I* enzyme, produced from 3 to 11 different restriction fragments, none of which are repeated in all fungi which indicates that the restriction sites for *Cla I* in the IGS region are more than one and which are located in different position in each of fungal DNA fragments. This enzyme can be key to differentiate fungal strains, because the 6 strains, where as the GS strain has bands between 200 and 250 bpand no the 300 bp fragment, in contrast GH1 strain has 200 and 300 bp fragments but not the 250 bp one, the PSH and AA20 strains have three bands but with different size becausePSH strains has a band of 500 bp which is absent in AA20, the ESH strain has a band of 250 bp and the PSS has only a band of 300 bp.

Digestion of the IGS fragments employing *Eco RI* produced fragments patterns with 3 to 16 fragments, but a band of 300 bp was commoninall strains, these results suggests that there is two restriction sites present in the same position in all fungal strains (TABLE 3). The GS and GH1 strains have a great similarity, but it is possible to identify them from each other, because GS has a 700 bp band which is no present in GH1. The PSH and AA20 strains also have similarity, the difference is based in two bands (200 and 700 bp) present in PSH and absent in AA20 strains. PSS and ESH strains are easy to distinguish from the rest of the strains because one had three bands and the other two bands with a band (300 bp) in common.

Digestion with *Hind III* produced 4-18 bands and none fragments pattern wasequal; the results are similar to those obtained with *Cla I*, using these fragments is easy to differentiate the control strain (AA20) because it presents only 4 bands. GH1 and GS strains have a similar fragments patterns but GH1 strains has 5 different bands (80, 130, 250, 550, 850 bp). The PSH strains differ from other fungal strains because it does not have a band of 550 bp present in GH1, and PSH has 6 frag-

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TABLE 3 : Restriction fragments weight (pb) in Amplified DNA from IGS Region in tannase producer fungi strains.

Fungalstrains	Cla I	EcoR I	Hind III	Pst I
PSS	400,300, 150	700, 500, 300	500, 300	400, 300
ESH	350, 250, 100	600, 300	500, 250	700, 490, 400, 300, 290, 220, 140, 100, 90, 50, 30
GS	600, 400, 350, 250, 200, 150, 120, 90, 50	700, 550, 400, 390, 300, 290, 250, 200, 190, 120, 100, 90, 50	500, 420, 400, 320, 300, 210, 190, 160, 120, 100, 90, 50	700, 600, 500, 490, 400, 350, 300, 290, 220, 200, 180, 140, 120, 90, 40, 20
PSH	700, 300, 250, 200	900, 700, 400, 390, 300, 290, 250, 200	900, 550, 420, 320, 300, 250	800, 500, 490, 400, 350, 300, 290, 220, 180, 140, 120, 100, 90, 40, 20
GH1	900, 600, 400, 300, 210, 200, 110, 100, 90, 50, 20	900, 600, 550, 500, 400, 390, 300, 290, 250, 200, 190, 150, 120, 100, 90,	900, 850, 550, 500, 420, 400, 320, 300, 250, 210, 190, 160, 130, 120,	900, 800, 600, 500, 490, 400, 350, 300, 290, 220, 200, 180, 140, 100,

ments while GH1 has 18 fragments. The PSS and ESH strains present only 2 fragments which make them easy to distinguish from the other fungal strains (TABLE 3).

The use *Pst I* enzyme for digestion of IGS fragments, proportioned from 2 to 16 fragments, being the 300 bp fragment more repeated in all strains, this indicates that all fungi studied share restriction sites. With this enzyme as the other three enzymes the GH1 and GS strains show great similarities, but GH1 did not exhibit two bands (120 and 700 bp) present in GS, but this have not the 100 bpb and present in GH1, this patternis similarity to that obtained with the PSH strain with the difference that GH1 contains bands of 100 and 120bp present in one strain but absent in other. The ESH strains present two bands 30 and 50 bp which were absent in the others strains, on the other hand, the PSS and AA20 (control) strains differs from the remainder, for the number of bands since they only have 2 and 3 fragments respectively. Edel et al. (1995) compared three methods for the molecular characterization of *Fusariumoxysporum* strains, and with enzymatic digestion of amplified regions obtained between 3 and 20 fragments using anenzyme which recognize restriction sites of four base pairs, where as when digestion was performed with an enzyme which recognize restriction sites of 6 base pairs, only one restriction site in the IGS fragment was observed, this is possible because 6 bases-restriction enzymes is less likely to cut. Results from the restriction of IGS region suggest that it is possible to identify the six fungal strains using four restriction enzymes; being the more effective pattern that obtained with the *Cla I* enzyme because fewer but informative fragments were obtained, allowing avoid confusion, in addition from all fungal strains presented at least a different band, in contrast, the other three enzymes offered a higher number of fragments but some with similar size, making difficult to identify them from each other with the naked eye.

Genetic relationships among tannase-producer fungal strains

The genetic relationship among fungal strains was estimatedusing restriction patterns of the ITS region (Figure 5). The results shown, that PSH and GH1 both *Aspergillusniger* strains are very close, both strains were isolated from different host, PSH strain was isolated from leaves of *Pinuscembroides* while GH1 strain from leaves of *L.tridentata*[4]. mention that these two

strains presented similar morphological characteristics, however, when a physiological characterization was performed both strains were different from each other. In addition, it was observed that the AA20 strain (control) has a high similarity with these two strains, although this strain was isolated from very different host (coffee plants), and from a very different geographical area (Jalapa, Veracruz). *Aspergillusterricola* PSS strain was located far away from the rest of the strains, which was expected since it is a differents pecie. In addition, PSS morphology and physiological behavior is very different to the *A. niger* strains.

The band patterns obtained after the digestion of

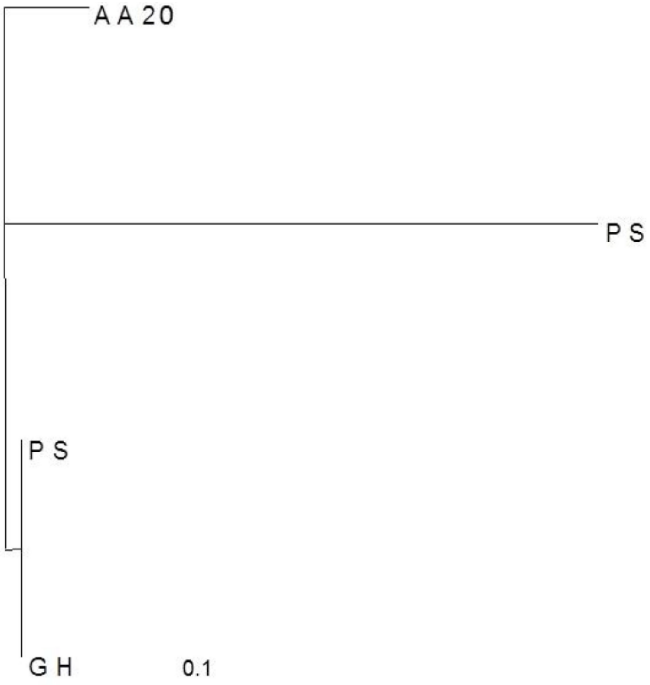

Figure 5 : Genetic relationship among tannase-producer fungal strains using restriction patterns of ITS bands.

IGS fragments (Figure 6) showed some inconsistencies with taxonomic relationships. Thus, the PSS strain (*A.terricola*) was very closeto *A. niger* AA20 strain (control) although, these two strains were isolated from different host, geographic area and present dissimilar morphological characteristics. PSH and GH1 strains were integrated in the same group (*A. niger*). There are morphological differences between GS (*A. fumigatus*) and GH1 (*A.niger*) strains which belong to different species. In general, a possible explanation to the inconsistency taxonomic results is the presence of many bands in the restriction pattern which difficult to assess identify presence of bands, it was especially difficult when very small bands were observed. Furthermore, IGS regions are most repeated in the genome and are more likely to accumulate polymorphism (mutation, inversions, translocations, etc.) making it harder to use them for estimation of the genetic relationships among species, strains or individuals, however, are excellent markers for differentiate between closely related individuals because containing high polymorphism levels.

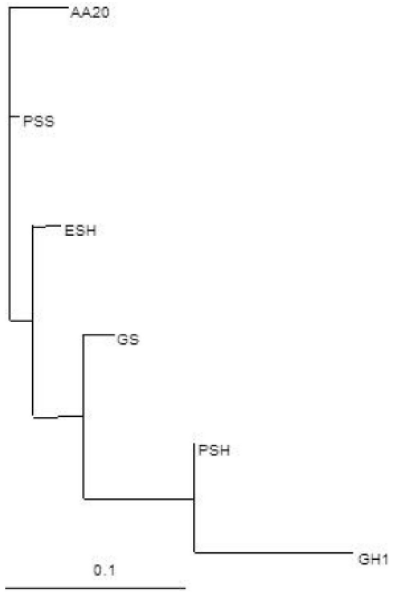

Figure 6 : Genetic relationship between tannase-producing fungal strains using digestion of IGS bands.

The polymorphism detected by RAPD was similar to that observed with digestion of IGS fragments. Dendrogram analysis is presented in Figure 7. Similarly to IGS data, the RAPDs dendrogram grouped PSH and GH1 strains which is in consistent with the taxonomic category. But again AA20 strain was placed in another group, despite belonging to the same species, *Aspergillusniger*. Since the three different polymorphism detection systemsused (ITS, IGS and RAPDs) have target different sequences which are expected to have different magnitudes of changes during the evolutionary process, all polymorphism results were used to group the fungal strains (Figure 8). The resulted dendrogram showed more consistences to the fungal taxonomy categories, because AA20, PSH and GH1 strains belonging to *Aspergillusniger*s pecie were placed in the same group. Furthermore ESH and GS belonging to *Aspergillusornatus* and *Aspergillusfumigatus*, respectively, were placed in the same group, but with a considerable distance between

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them. The consistency of this diagram shows the need to use different polymorphism detection systems, to elucidate genetic and evolutionary relationships.

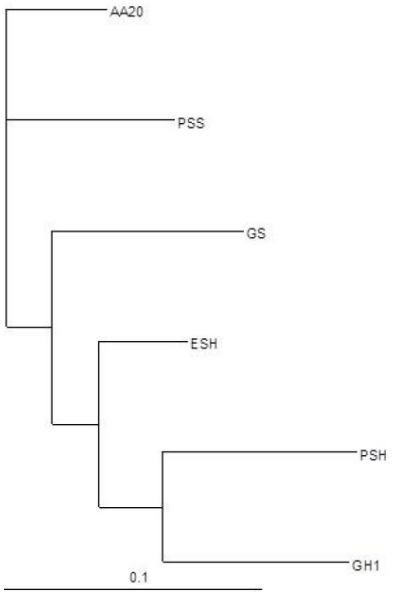

Figure 7 : Genetic relationships between fungal strains producing tannase using three detection polymorphism systems.

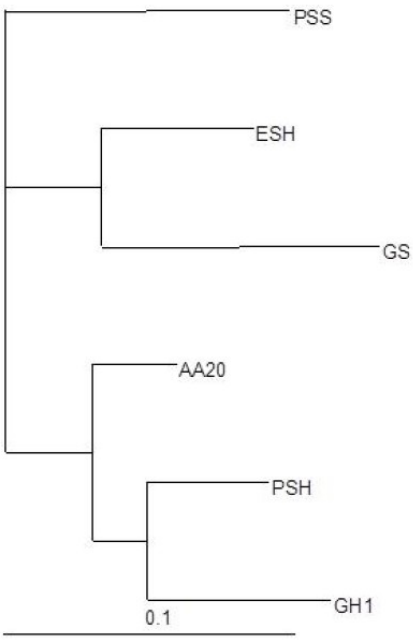

Figure 8 : Genetic relationship among tannase-producing fungal strains using three polymorphism detection systems.

The RAPD's and IGS systems provided similar number of bands, presence of bands with different size permitan easily characterization of fungal strains, while ITS band pattern was somewhat complicated because it was possible to amplify a band of similar size from all fungal strains, but these strains only may be differentiated from each other, by ITS fragment sequencing orrestriction analysis. On the other hand, ITS and IGS amplifications are reproducible, whereas with RAPD's results may change which is one disadvantage of this technique because is very sensitive to experimental conditions, which limits its adoption[11,14].

On the other hand, it was not possible to amplify the ITS region of two strain (GS and ESH) with the primers used, one possible explanation is mutation of the primer annealing site of these fungal strains which does not allowed primer coupling for PCR amplification. When restriction of the IGS fragments was performed, it was observed a pattern with many bands which without a digital imaging equipment is difficult to assess, however this can be remedied in part by using restriction enzymes which act on 6 o 8-base restrictionsites. The combination of the three systemspermitted a better molecular characterization of tannase-producing fungal strains, more polymorphic bands allowed a dendrogram consistent with the taxonomic classification, but not with host or geographic location. Three strains belonging to *Aspergillusniger* were placed in a group, *Aspergillusfumigatus* and *Aspergillusornatus*in another, while *Aspergillusterricola*is separate from the last two groups. This shows the power of DNA polymorphisms to access genetic relationships among fungal strains, but also indicates the need to combine several different genetic fingerprint systems.

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