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A N   I N T E R N A T I O N A L   J O U R N A L

Use of RAPD Molecular Markers on Differentiation of Brazilian and Chinese *Ganoderma lucidum* Strains

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ABSTRACT

The aim of this work was to analyze the Brazilian and Chinese strains of Ganoderma lucidum *with molecular RAPD markers. A similarity matrix was elaborated and the RAPD profiles of* G. lucidum *strains were also compared to two other* Ganoderma *spp:* G. applanatum *and* G. lipsiense *in order to produce genetic similarity among the species. Based on the primers used, it was possible to determine that the Brazilian strains and Chinese strain CC-22 are alike. The method and the primers selection showed to be appropriate for the genetic identification of* G. lucidum *strains, enabling them to be improved and used in research, as well as in the world market.*

Key words: *Ganoderma lucidum*, genetic diversity, RAPD

INTRODUCTION

Ganoderma lucidum (Fr.) Karst. (Ganodermataceae) is a very popular basidiomycete on the traditional Chinese medicine, and is also used in other countries as Japan and the United States as food supplement. In Brazil, though it is little exploited, this fungus is known as King Mushroom, eaten as food supplement (Mau et al., 2001). Studies carried out with *G. lucidum* reveal many biological properties, such as anti-tumor, anti-inflammatory, antiviral (anti-HIV) and antibacterial activity. Its pharmacological effects have been studied in the treatment against many human diseases such as hepatitis, hypertension, heart problems, arthritis, bronchitis, tumors, diabetes, insomnia, and others (Kim, 1987; Nogami, 1987; Kim et al., 1995; El-Mekkawy et al., 1998; Eo et al., 1999; Wasser and Weis, 1999; Hsieh et al., 2005; Lakshmi et al., 2006; Tang et al., 2006; Chu et al., 2007; Li et al., 2007; Zhu et al., 2007).

The taxonomy of the genus is traditionally based on the morphological characteristics. Though they are really useful for differing species, there are still difficulties for distinguishing the close groups such as the populations or strains of the same species. According to Zheng et al. (2007), environmental factors, variability, interhybridization and morphological propensity make the accurate identification hard for *Ganoderma* species. However, the new techniques using molecular markers has turned easy the identification not only for *Ganoderma* species, but also for other organisms which has the same taxonomic difficulties. The use of DNA as

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identification tool allowed to differentiate the subgroups, as the various *G. lucidum* strains, making easy to distinguish mainly the commercial strains and strains of industrial interest (Zheng et al., 2007).

Several techniques for molecular studies have been used for analyzing the genetic diversity in basidiomycetes, such as isoenzymes (Lan et al., 1998), AFLP (*Amplified Fragment Length Polymorfism*) (Qi et al., 2003), RFLP (*Restriction Fragment Length Polymorfism*) (Park et al., 1996), ITS (*Internal Transcribed Spacers*) (Kindermann et al., 1998) and RAPD (*Random Amplified Polymorphic DNA*) (Wang et al., 2003). Among these techniques, RAPD is still one of the cheapest and quickest methods for accessing the variability at DNA level, being especially useful on intraspecific analysis. These markers have the advantage of amplifying both regions of the genome which may be transcript/translated, and non-coding regions. This is important when the objective is to evaluate the variation along the biggest part of the species genome (Williams et al., 1990; Ferreira and Grattapaglia, 1996; Ro et al., 2007). On the other hand, RAPD has

limitations for its low experimental reproducibility, although this problem may be overcome with the use of many primers and with more strict criteria in analyzing the results (Carvalho and Vieira, 2001).

This work aimed to select the RAPD molecular markers for *Ganoderma lucidum*, evaluating the genetic similarity pattern between the Brazilian and the commercial Chinese strains.

MATERIAL AND METHODS

Ganoderma lucidum strains

Four *G. lucidum* strains were used: two Brazilian (identified as CC-144 and CC-157) and two Chinese commercial strains (CC-22 and CC-63) (Table 1). These are available in the Germplasm Bank of Mushrooms for Human Use of Embrapa Genetic Resources and Biotechnology (Brasilia, DF, Brazil). Besides these, the samples of two other Ganoderma strains were used: *G. applanatum* and *G. lipsiense* (originated from China), in order to establish the genetic comparisons at genus level.

Table 1 – Origin of the *G. lucidum* strains used in the study.

Strain	Local	Date of Collection
CC-22	China	19/02/2004
CC-63	China	10/02/2006
CC-144	Brasília, Brazil	10/02/2006
CC-157	São Paulo, Brazil	25/01/2006

DNA extraction

Fungi were taken from the matrix tubes and grown in Petri dishes containing PDA (20% Potato, 2% Dextrose, 1.5% Agar), for seven days in complete darkness at 28° C and RU 82%. Each isolate was transferred to PDA liquid medium at 28° C for two weeks. The medium with mycelium was centrifuged at 3,000 rpm for 10 minutes, washed with sterilized water and softly pressed on filter paper. Strains were transferred to Eppendorf tubes of 1.5 mL in which they were macerated in liquid nitrogen, treated with 1 mL of extraction buffer solution (1M Tris-HCl pH 8.0, 0.5M EDTA pH 8.0, 10% SDS, 5M NaCl) (Raeder and Broda, 1985) and incubated for one hour at 65° C. Then a phenolic solution was added, softly mixing the phases and centrifuged at 10,000 rpm for 15 minutes. The aqueous phase was transferred to new Eppendorf tubes with a solution of

chlorophorm:isoamilic acid (24:1), mixed and centrifuged as above. The supernatant was taken in the tubes with one volume of NaCl (1M) and two volumes of ethanol (chilled to -20° C). After centrifugation at 15,000 rpm for 15 minutes, the supernatant was disposed and the precipitate was washed with ethanol (70%). The microtubes were dried and the material was resuspended in 20µL of TE buffer solution (1M Tris-HCl pH 8.0, 0.5M EDTA pH 8.0). The samples had their DNA concentration quantified on agarose gel 1.5% and stocked up at -80° C.

RAPD analysis

The DNA from the strains was amplified through RAPD according to Williams et al. (1990), using 48 arbitrary primers from Operon Technologies Inc. (Alameda, CA, USA), with average size of 10 bases: OPAB-02, OPAB-14, OPAB-18, OPAB-19,

OPA-02, OPA-05, OPA-07, OPA-11, OPA-12, OPA-17, OPA-18, OPB-02, OPB-05, OPB-06, OPB-09, OPB-10, OPB-11, OPC-02, OPC-18, OPC-19, OPD-02, OPD-04, OPD-07, OPD-20, OPE-04, OPE-06, OPE-08, OPF-01, OPF-02, OPF-06, OPF-08, OPF-12, OPG-04, OPG-15, OPH-12, OPO-15, OPO-19, OPR-02, OPR-08, OPR-09, OPR-10, OPR-12, OPR-19, OPR-20, OPU-01, OPV-08, OPW-18 and OPX-20. The reactions were carried out in a thermocycler PTC-100 (MJ Research Thermal Cycler), with the use of a program following the due steps: (1) DNA melting at 96°C for 3 minutes and (2) at 92° C for 1 minute; (3) primer annealing at 35° C for 1 minute; (4) molecule extension with Taq polymerase (provided by Amersham Pharmacia Biotech) at 72° C for 2 minutes; (5) 40 cycles following steps 2-4; (6) final step of the 5 minute extension at 72° C in order to finalize the amplified products. For amplifying the samples, the following compounds were used for a total volume of 13 µL: 3.0 µL genomic DNA at 3.00 ng/µL; 4.92 µL Milli-Q water; 1.3 µL buffer solution 10X for Taq DNA polymerase; 1.04 µL dNTP 2.5 mM; 1.04 µL BSA 2.5 mM; 1.5 primer 10ng/µL and 0.2 µL Taq DNA polymerase. The samples of amplified DNA were put on agarose gel 1.5% (120 volts for 4h), on TAE buffer solution 1X, using the 1kb DNA ladder molecular mass marker (Life Technologies, Cergy-Pontoise, France) as reference. This gel was stained with etide bromide and observed through transilluminator under UV light and photographed with Stratagene's camera for documentation Eagle Eye II. Three repetitions of the RAPD were made, the first only with *G. lucidum* strains, other similar, with the addition of *G. applanatum*, and a third one, using *G. lipsienses.*

Post-RAPD Analysis

The primers which presented better band pattern were used for building a similarity index among the pairs of isolates, based on the Jaccard coefficient (Sj,j) (Dias, 1998), designating 1 for the presence of band and 0 for the absence of band in the gel. The Jaccard coefficient (Sij) among the pairs of strains is given by Sij = a / (a+b+c), in which "a" is the number of positive marks between the i and j isolates, while "b" and "c" are the number of loci present in only one of the isolates. Sij values close to 1.0 indicate high genetic similarity among the strains. In order to represent graphically the genetic divergence patters, the similarity matrix was submitted to a group analysis like UPGMA (Unweighted Pair-Group Method Analysis), using the NTSYS program (Numerical Taxonomy and Multivariate Analysis System) version 2.2. In this method, the criteria used for generating the groups is the average of the distances among all the pairs of items from each group as the denomination itself informs. The inter-group distance is the average of the paired distances of members from both groups (Dias, 1998).

The binary data generated from the observations in gel were used in a computer procedement of re-sampling on loci (bootstrap). This analysis consists in resampling the loci of the data matrix, with reposition, recalculating the desired statistics several times, testing the stability of the genetic distance obtained through RAPD. Four thousand repetitions were made following the Monte Carlo permutation method. The Monte Carlo permutation method produces simulated dissimilarity values, obtained from data matrix generated through the relocation of the original values of samples (Manly, 1997). Only when the similarity index of the original data (i.e., with no simulation) is significantly higher than the index obtained after permutations, the genetic similarity is considered significant (significance level adopted, α = 0.05). Permutations were made with the software RandMat version 1.0 for Windows.

RESULTS AND DISCUSSION

All the strains presented positive result on forming the bands on agarose gel, according to the technique for RAPD analysis. From the 48 primers tested, 20 did not amplify or present good amplification pattern. The other 28 primers showed good amplification pattern and presented a total of 512 loci, 323 of them as polymorphs. The average number of loci found was 18.28, being 11 the minimum and 26 the maximum number of loci per primer (Table 2).

Table 2 – Relation of numbers of efficient loci and polymorphic loci per primer for RAPD analysis of *Ganoderma lucidum* strains.

Primers	5' – 3' sequence	number of loci	number of polymorphic loci
OPAB-14	AAGTGCGACC	17	14
OPA-05	AGGGGTCTTG	17	11
OPA-07	GAAACGGGTG	24	20
OPA-11	CAATCGCCGT	18	10
OPA-12	TCGGCGATAG	24	14
OPB-05	TGCGCCCTTC	26	24
OPB-06	TGCTCTGCCC	16	12
OPC-18	TGAGTGGGTG	21	16
OPC-19	GTTGCCAGCC	16	13
OPD-04	TCTGGTGAGG	11	06
OPD-20	ACCCGGTCAC	19	17
OPE-06	AAGACCCCTC	19	10
OPE-08	TCACCACGGT	16	10
OPF-01	ACGGATCCTG	17	04
OPF-02	GAGGATCCCT	12	05
OPF-08	GGGATATCGG	17	09
OPF-12	ACGGTACCAG	20	09
OPG-15	ACTGGGACTC	22	10
OPH-12	ACGCGCATGT	17	10
OPO-15	TGGCGTCCTT	18	04
OPO-19	GGTGCACGTT	17	10
OPR-08	CCCGTTGCCT	15	08
OPR-09	TGAGCACGAG	19	10
OPR-12	ACAGGTGCGT	16	04
OPR-19	CCTCCTCATC	14	13
OPR-20	ACGGCAAGGA	17	12
OPV-08	GGACGGCGTT	24	17
OPW-18	TTCAGGGCAC	23	21
TOTAL		512	323

The presence and absence of bands were registered on worksheet and used for generating a similarity index. Some bands were not as legible as the other ones, but this did not affect the structure of the generated dendrogram, because only the bands which remained after the repetitions were considered in the study.

The first comparisons made based on the 28 primers indicated that the Brazilian strains CC-144 and CC-157 were the closest ones between themselves, with Jaccard value 0.798. Comparing with the Chinese strains, the group formed by the Brazilian strains was closer to the CC-22 strain (Jaccard = 0.721), presenting smaller genetic similarity with the CC-63 (Jaccard = 0.52)

The bootstrap analysis, which is used in population genetics in order to evaluate the stability of groups obtained from the genetic distances and to establish the confidence intervals in unfolding the models of variance and populational structure (Weir, 1990; Meyer, 1995; Hillis et al., 1996; Manly, 1997) revealed that CC-63 was the only strain whose RAPD showed to be uncertain for determining the genetic similarity among the studied strains. The dendrogram showed that the genetic divergence of CC-63 with the other ones was in the uncertainty area

generated by the simulated re-sample analysis (Jaccard 0.43 to 0.6). Result indicated that the point of genetic divergence between the CC-63 and the other strains could oscillate inside the informed area. In the other strains, the genetic similarity and the points of divergence were statistically significant, showing stable result among CC-22, CC-144 and CC-157.

These results showed that the CC-22, CC-144 and CC-157 *G. lucidum* strains could be differentiated through the RAPD with the primers listed in Table 2. Hseu et al. (1996) studied *G. lucidum* strains as the RAPD tests and observed the sturdiness of their markers. According to the author, the technique could be applied on different strains and populations of a same species, being of low accuracy when dealing with big taxonomic groups. The RAPD may not be very useful when applied to determine very elaborated phylogenies, evolving big taxonomic groups, but its results are recognized when specific groups are studied (Duncan et al., 1993; Hamelin et al., 1993; Manulis et al., 1994; Laroche et al., 1995; Gomes et al., 2009; Stringari et al., 2009; Gonçalves et al., 2010).

Ro et al. (2007) obtained good results when they used RAPD aiming to differentiate the strains of

Pleurotus eryngii. The technique has been used in the studies with *Verticillium fungicola*, with excellent results (Del Carmen et al., 2002; Largeteau et al., 2006) and by other researchers for works with many other organisms, always differentiating very close taxonomic groups (Grazziotin et al., 2005; Muok et al., 2007; Tang et al., 2007; Rajwana et al., 2008; Solouki et al., 2008; Venkatachalam et al., 2008; Tsvelikas et al., 2009). The primers used in this study, however, were not enough to measure with accuracy the genetic distance of the strain CC-63 (Chinese strain), and it is suggested could be use other primers for analyzing or complete the differentiation.

The RAPD profiles of the *G. lucidum* strains were also compared to *G. applanatum* and *G. lipsiense*. The analysis with the 28 used primers resulted in 590 loci for *G. applanatum*, from which 440 were polymorphs when compared to *G. lucidum* strains. *G. lipsiense* revealed 597 loci, 446 of them being polymorphs when compared with the loci of the *Ganoderma lucidum* strains. These results were compared in order to establish a genetic similarity at genus level among the studied isolates (Fig. 1). Figure 2 shows the distinct RAPD profiles of a sample of isolates in agarose gel.
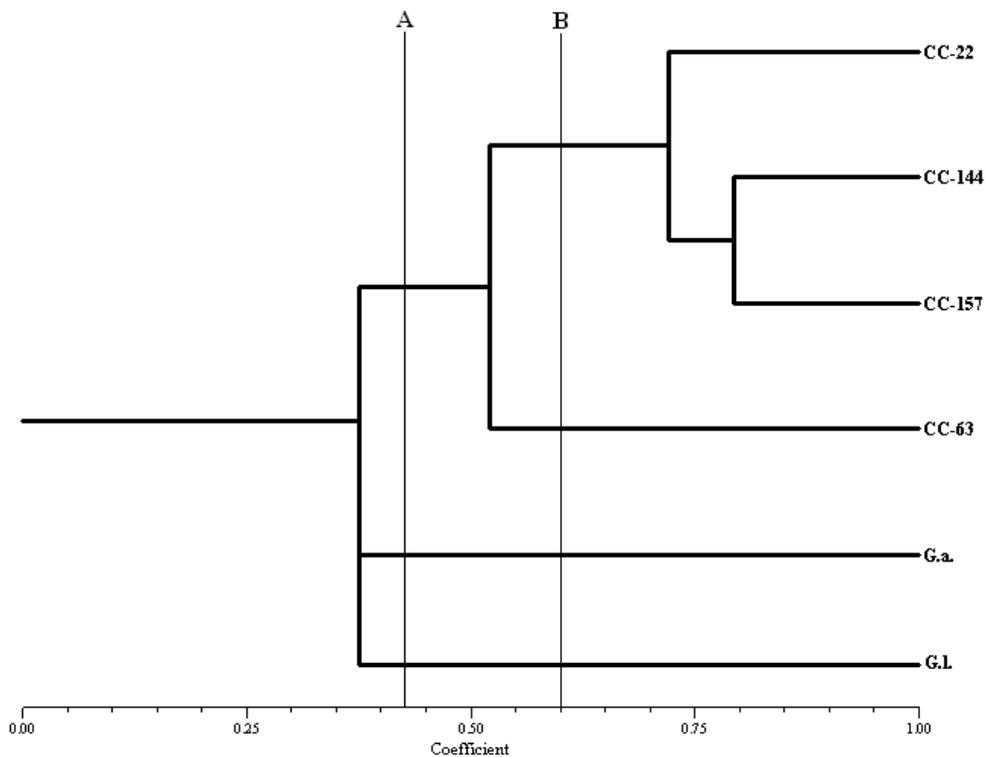
Figure 1 – Dendrogram showing the genetic similarity between the Chinese strains (CC-22 and CC-63) and the Brazilian strains (CC-144 and CC-157) of *G. lucidum, G. applanatum* (G.a.) and *G. lipsiense* (G.l.) after RAPD analysis. The area present between the columns A and B determine an imprecision gap on genetic analysis, informing that the genetic distance between CC-63 and the other strains may oscillate inside the observed boards.
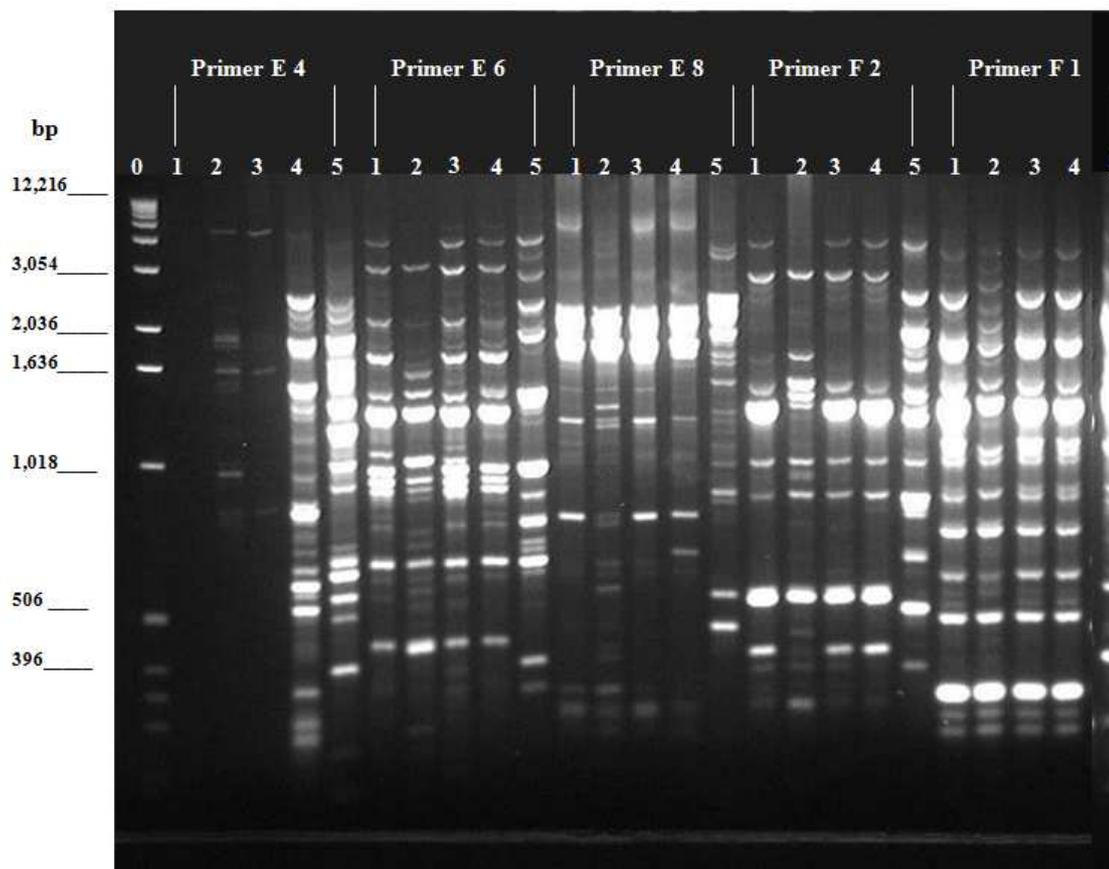
Figure 2 – RAPD-PCR comparison between brazilians and chineses *Ganoderma lucidum* strains. Lanes: 0, 1kbp DNA ladder; 1, CC-22; 2, CC-63; 3, CC-144; 4, CC-157; 5, *Ganoderma applanatum* (included to ensure the consistency of the bands). Primers used: OPE-4, OPE-6, OPE-8, OPF-1 e OPF-2.

The results showed that both *G. applanatum* and *G. lipsiense* were more genetically distant when compared to the *G. lucidum* strains (Jaccard 0.371), as expected, for being strains of different species. The other *G. lucidum* strains remained stable, and CC-63 remained into the imprecision area. Thus, the RAPD analysis highlighted the difference between the species and could be applied to differentiating strains of the same species. Aiming to develop the genetic patterns obtained from RAPD techniques, there are only few others studies (such as the present one) dealing with *G. lucidum* (Hseu et al., 1996; Singh et al., 2005; Ro et al., 2007).

The major difficulty in applying this technique in studies with systematic relies on the extreme sensitivity of RAPD. Genetic profiles may suffer variations depending on the way the analysis is carried out. However, it is possible to obtain re-sampling results through the repetitions, once patterns start being designed through this process. There several studies on RAPD, but they converge on the fact that it would be used when a pattern was found through repetitions with strict criteria when evaluating the results (Ellsworth et al., 1993; Muralidharan and Wakeland, 1993; Leal et al., 1994; Tommerup et al., 1995; Hseu et al., 1996; Moore et al., 2001; Utomo et al., 2005; Ercisli et al., 2007; Jones et al., 2008; Kim et al., 2008). In this study, the analysis was repeated three times, until a pattern on the profile of the bands was achieved.

The observations on genetic similarity between the Brazilian and Chinese strains contributed the molecular chart of the genus *Ganoderma*, suggesting genetic relationship among the species

and strains, regardless the geographical distance and allowing the use of the RAPD in basic and applied research, as well as in industry, with low costs.

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