Plant Gene 6 (2016) 43–47

Contents lists available at ScienceDirect

Plant Gene

journal homepage: www.elsevier.com/locate/plantgene

TRAP and SRAP markers to find genetic variability in complex polyploid *Paullinia cupana* var. *sorbilis*

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ARTICLE INFO

Article history:
Received 7 July 2015
Received in revised form 16 February 2016
Accepted 11 March 2016
Available online 16 March 2016

Keywords:
Guarana plant
Clonal Cultivar
Germplasm
Amazon germplasm
Molecular marker

ABSTRACT

The guarana plant (*Paullinia cupana* var. *sorbilis*) is a polyploid rich in natural caffeine, used as a source for producing industrial soft drinks. Embrapa Western Amazon maintains an Active Germplasm Bank (BGA) with 270 clones, which represents the genetic basis of the species conservation and breeding programs. The BGA evaluations conducted using phenotypic traits and Random Amplified Polymorphic DNA (RAPD) markers indicated low genetic variability. Therefore, the objective of this study was to analyze the genetic diversity of the clonal germplasm of the guarana plant using Target Region Amplification Polymorphism (TRAP) and Sequence-Related Amplification Polymorphism (SRAP) markers. Sixty clones of the guarana plant were analyzed; 18 were cultivars, eight were similar clones according to morpho-agronomic traits, and 34 were clones of a different origin. The percent polymorphism found was 79% for TRAP and 74.5% for SRAP. The Polymorphic Information Content (PIC) values for both markers ranged from 0.29 to 0.37, with an amplified fragment size between 100 and 800 bp. No genotypes with genetic similarity of one were found in the three germoplasm samples analyzed, which ruled out the possibility of the occurrence of duplicates. In the cluster analysis, the dendrogram generated by the SRAP marker added three additional groups when compared with TRAP and distinguished two genotypes in the sample comprising morpho-agronomically similar clones. The TRAP and SRAP markers yielded complementary information on the genetic variability of the guarana germplasm. Therefore, the combination of the two markers has the potential to broaden genetic characterizations and facilitate parental selection in breeding programs.

1. Introduction

The guarana plant (*Paullinia cupana* var. *sorbilis* (Mart.) Ducke) is a polyploid of complex origin with 2n = 210 and a genome size of 1C = 11.4 pg (de Freitas et al., 2007). The most common chromosome number is 2n = 24 in karyotyped species of the genus *Paullinia* (Urdampilleta et al., 2007; Solís-Neffa and Ferrucci, 2001), whereas the genome size recorded for the subfamily Paullinieae ranges from 1C = 0.305 pg to 2.710 pg (Coulleri et al., 2014). There is no record indicating whether the species evolved from the duplication of a single genome or from a combination of genomes nor of its relationships with the genetic variability present in the guarana crop.

The socioeconomic relevance of this plant native to the Amazon rainforest is associated with the high caffeine content in the guarana seeds. Most of the production is used by the soft drinks industry, and the rest is marketed in the form of syrup, stick, powder, and extracts (Antonelli-Ushirobira et al., 2010). The main problems of the crop are low yield and diseases caused by fungi. Embrapa Western Amazon

Abbreviations: A, adenine; AFLP, Amplified Fragment Length Polymorphism; AGB, Active Germplasm Bank; bp, base pairs; BRS, Brazil; BRSCG, Brazil guarana plant clone; C, cytosine; C, DNA content; °C, degree Celsius; CIR, clone from Iranduba City; CMA, clone from Manaus City; CMU, clone from Maués City; CTAB, Cetyl trimethylammonium bromide; DNA, Deoxyribonucleic acid; dNTP, deoxyribonucleoside triphosphate; Embrapa, Brazilian Agricultural Research Corporation; EST, Expressed Sequence Tags; Fig., Figure; G, guanine; ISSR, Inter-Simple Sequence Repeat; kb, kilobase(s) or 1000 bp; mM, millimolar or 10^{-3} mol/m^3; μM, micromolar or 10^{-6} mol/m^3; n, gametic or haploid number; ng, nanogram; PCR, Polymerase chain reaction; Pg, pictogram; pM, picomolar or 10^{-9} mol/m^3; PIC, Polymorphic Information Content; T, thymine; Taq, polymerase from the thermophilic bacterium; TRAP, Target Region Amplification Polymorphism and; RAPD, Random Amplified Polymorphic DNA; SRAP, Sequence-Related Amplification Polymorphism; SSR, Simple Sequence Repeat; U, enzyme unit; UTR, untranslated regions; Var, variety.

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http://dx.doi.org/10.1016/j.plgene.2016.03.005

maintains an Active Germplasm Bank (Banco Ativo de Germoplasma — BGA) with 270 clones to provide genetic variability for breeding and conserving the species for future demands (Atroch et al., 2012). Eighteen cultivars have already been selected and recommended for the commercial production of seeds in the clone competition program (Souza et al., 2012).

The use of germplasm has been guided by the knowledge of the variability in phenotypic traits and molecular markers. Nascimento-Filho et al. (2001) observed low genetic divergence between clones using various techniques based on agronomic phenotypes. A low degree of variation with no association with the collection sites was also detected in the characterization of germplasm using a Random Amplified Polymorphic DNA (RAPD) marker (Atroch et al., 2012). No identical genotypes were found in the characterization of 15 cultivars using 10 microsatellite loci generated from genomic libraries and the transcriptome of the guarana fruit.

The availability of genomic sequences enabled the development of multilocus markers targeting more specific regions, among them the Target Region Amplification Polymorphism (TRAP) and Sequence-Related Amplified Polymorphism (SRAP) markers. The TRAP marker is based on the combination of a fixed primer, a sequence designed from Expressed Sequence Tags (ESTs), and an arbitrary primer. The fixed primer will anneal to a given expressed region of the genome during the PCR reaction, and the polymorphism generated by the combination of the two primers will be associated with a specific gene (Hu and Vick, 2003). The SRAP marker also combines two primers, each containing random sequences with a common motif consisting of CCGG in the forward primer and AATT in the reverse primer. These motifs are essential to generate a polymorphism associated with gene regions, considering that the regions coding for proteins contain GC-rich codons and AT-rich untranslated regions (UTRs) (Li and Quiros, 2001).

The TRAP and SRAP markers amplify different regions, and both have the advantages of being a simple technique, as well as providing high yield, reproducibility, and the possibility to sequence their products (Poczai et al., 2013). Markers with higher polymorphism can further elucidate the genetic variability of this polyploid of complex inheritance found in the Amazon region in the cultivated form only. The objective of this work was to evaluate the potential of TRAP and SRAP markers to genetically characterize the guarana germplasm.

2. Materials and methods

2.1. Germplasm sample

The material analyzed represents a sample from the guarana BGA comprising 18 clonal cultivars (BRS), eight clones with similar morpho-agronomic traits (Nascimento-Filho et al., 2001), and 34 clones from three different collection sites in the state of Amazonas in Brazil: Manaus (CMA), Maués (CMU), and Iranduba (CIR) (Table 1).

Table 1
Germplasm accessions analyzed in this study.

Germplasm accessions	Germplasm types and origins	Germplasm accessions	Germplasm types and origins
BRS-CG372	Clonal cultivar	CIR 819	Origin Iranduba
BRS-CG505	Clonal cultivar	CMU 381	Origin Maués
BRS-CG608	Clonal cultivar	CMU 385	Origin Maués
BRS-CG610	Clonal cultivar	CMU 389	Origin Maués
BRS-CG611	Clonal cultivar	CMU 500	Origin Maués
BRS-CG612	Clonal cultivar	CMU 502	Origin Maués
BRS-CG648	Clonal cultivar	CMU 607	Origin Maués
BRS-CG850	Clonal cultivar	CMU 609	Origin Maués
BRS-CG882	Clonal cultivar	CMU 614	Origin Maués
BRS-Maués	Clonal cultivar	CMU 617	Origin Maués
BRS-Manaus	Clonal cultivar	CMU 619	Origin Maués
BRS-Amazonas	Clonal cultivar	CMU 625	Origin Maués
BRS-Andirá	Clonal cultivar	CMU 623	Origin Maués
BRS-Luzéia	Clonal cultivar	CMU 628	Origin Maués
BRS-Marabitanas	Clonal cultivar	CMU 862	Origin Maués
BRS-Mudurucania	Clonal cultivar	CMU 872	Origin Maués
BRS-Cereçaporanga	Clonal cultivar	CMU 874	Origin Maués
BRS-Saterê	Clonal cultivar	CMU 899	Origin Maués
CMA-222[a]	Similar clones	CMU 908	Origin Maués
CMA-223[a]	Similar clones	CMU 932	Origin Maués
CMA-224[a]	Similar clones	CMU 948	Origin Maués
CMA225[a]	Similar clones	CMU 952	Origin Maués
CMA227[a]	Similar clones	CMA 436	Origin Manaus
CMA228[a]	Similar clones	CMA 498	Origin Manaus
CMA274[a]	Similar clones	CMA 514	Origin Manaus
CMA276[a]	Similar clones	CMA 186	Origin Manaus
CIR 196	Origin Iranduba	CMA 375	Origin Manaus
CIR 202	Origin Iranduba	CMA 604	Origin Manaus
CIR 215	Origin Iranduba	CMA 831	Origin Manaus
CIR 217	Origin Iranduba	CMA 838	Origin Manaus

[a] Clones with similar morpho-agronomic traits (Nascimento-Filho et al., 2001).

2.2. DNA extraction

The total DNA was extracted according to the recommendations in the 2% cetyl trimethylammonium bromide (CTAB) method (Doyle et al., 1990). The samples were diluted following DNA quantification with a Nanodrop spectrophotometer.

2.3. TRAP marker

The fixed primers were designed from ESTs deposited in the guarana genomic bank using the Primer3 software. The fixed primers were tested in pairs with arbitrary primers, and the five combinations with better resolution on an agarose gel and a higher number of polymorphic loci were selected (Table 2). The PCR reactions were standardized according to the protocol described by Hu and Vick (2003), with modifications for the current species. The final reaction volume was 15 μL at a 1× buffer concentration: 1.5 mM $MgCl_2$; 0.8 μM dNTPs; 50 ng of DNA; 2.25 pmol of fixed primer and 1.5 pmol of random primer; and 1 U of Taq polymerase (PROMEGA). The amplification program consisted of a denaturation step at 94 °C for 2 min and five cycles of 94 °C for 45 s, 35 °C for 45 s, and 72 °C for 1 min. These steps were followed by 35 cycles of 94 °C for 45 s, annealing temperature of each primer combination for 45 s, and 72 °C for 1 min and a final extension step at 72 °C for 7 min. The PCR products were separated in an UltraPure 2% agarose gel stained with ethidium bromide.

2.4. SRAP marker

Reaction standardization and amplification program optimization were adapted for the guarana plant. Eight primer combinations, described by Li et al. (2012), were selected in this study (Table 2). The final PCR reaction volume was 15 μL at a 1× buffer concentration: 1.5 mM $MgCl_2$, 0.8 μM dNTPs, 50 ng of DNA, 1 pmol of forward primer, 1 pmol of reverse primer, and 1 U of Taq polymerase (PROMEGA). The amplification program applied was as follows: 94 °C for 2 min, followed by five cycles of 94 °C for 30 s, 35 °C for 30 s, and 72 °C for 45 s, then an additional 35 cycles of 94 °C for 30 s, 50–60 °C for 30 s, and 72 °C for 45 s. The final extension was performed at 72 °C for 7 min. The PCR products were separated in an UltraPure 1.5% agarose gel stained with ethidium bromide.

2.5. Data analysis

A binary matrix coded for the presence (1) or absence (0) of amplified fragments was prepared for each marker. The Polymorphic Information Content (PIC) value of each primer combination and the mean for the marker were obtained with the PICcalc software (Nagy et al.,

Table 2
TRAP and SRAP primers used to analyze clones in the active germplasm bank of guarana.

Primer	Sequence (5′-3′)		Tm	Number of bands		P (%)[a]	PIC
				Total	Polymorphic		
TRAP1 (*Auxr/T13*)	F: TCATCACCCGCTTGTATG	A: GCGCGATGATAAATTATC	53	19	13	68	0.31
TRAP2 (*Auxr/T03*)	F: CACAGACCCCGCCTTATAAA	A: CGTAGCGCGTCAATTATG	52	32	31	97	0.36
TRAP3 (*Auxr/T13*)	F: CACAGACCCCGCCTTATAAA	A: GCGCGATGATAAATTATC	52	38	33	87	0.35
TRAP4 (*Cystf/T03*)	F: AGGAGGTGGTCATGGTCCTG	A: CGTAGCGCGTCAATTATG	54	22	12	55	0.29
TRAP5 (*Cyst/FT14*)	F: AGGAGGTGGTCATGGTCCTG	A: GTCGTACGTAGAATTCCT	55	25	18	72	0.35
SRAP1 (*ME02/EM05*)	F: TGAGTCCAAA**CCGG**AGC	R: GACTGCGTACG**AATT**AAC	53	16	13	81	0.37
SRAP2 (*ME04/EM03*)	F: TGAGTCCAAA**CCGG**ACC	R: GACTGCGTACG**AATT**GAC	52	17	14	82	0.37
SRAP3 (*ME05/EM01*)	F: TGAGTCCAAA**CCGG**AAG	R: GACTGCGTACG**AATT**AAT	51	14	10	71	0.30
SRAP4 (*ME05/EM03/*)	F: TGAGTCCAAA**CCGG**AAG	R: GACTGCGTACG**AATT**GAC	55	14	10	71	0.30
SRAP5 (*ME01/EM10*)	F: TGAGTCCAAA**CCGG**ATA	R: GACTGCGTACG**AATT**CAG	54	10	7	70	0.37
SRAP6 (*ME10/EM07*)	F: TGAGTCCTTT**CCGG**TCC	R: GACTGCGTACG**AATT**CAA	52	19	13	68	0.30
SRAP7 (*ME15/EM03*)	F: TGAGTCCAAA**CCGG**CAT	R: GACTGCGTACG**AATT**GAC	50	18	12	66	0.29
SRAP8 (*ME19/EM07*)	F: TGAGTCCAAA**CCGG**TGC	R: GACTGCGTACG**AATT**CAA	53	14	11	78	0.37

[a] P: Polymorphism.

2012). Polymorphism levels by pair of primers were calculated using the arithmetic mean of the number of polymorphic loci per total number of loci (Table 2). The dendrogram was generated by the Unweighted Pair Group Method Averages (UPGMA) method with the NTSYSpc-2.02 software (Rohlf, 2000) using the Dice genetic similarity matrix.

3. Results

3.1. TRAP marker

The five primer combinations of the TRAP marker yielded 136 bands with 79% polymorphism. The total number of bands per combination

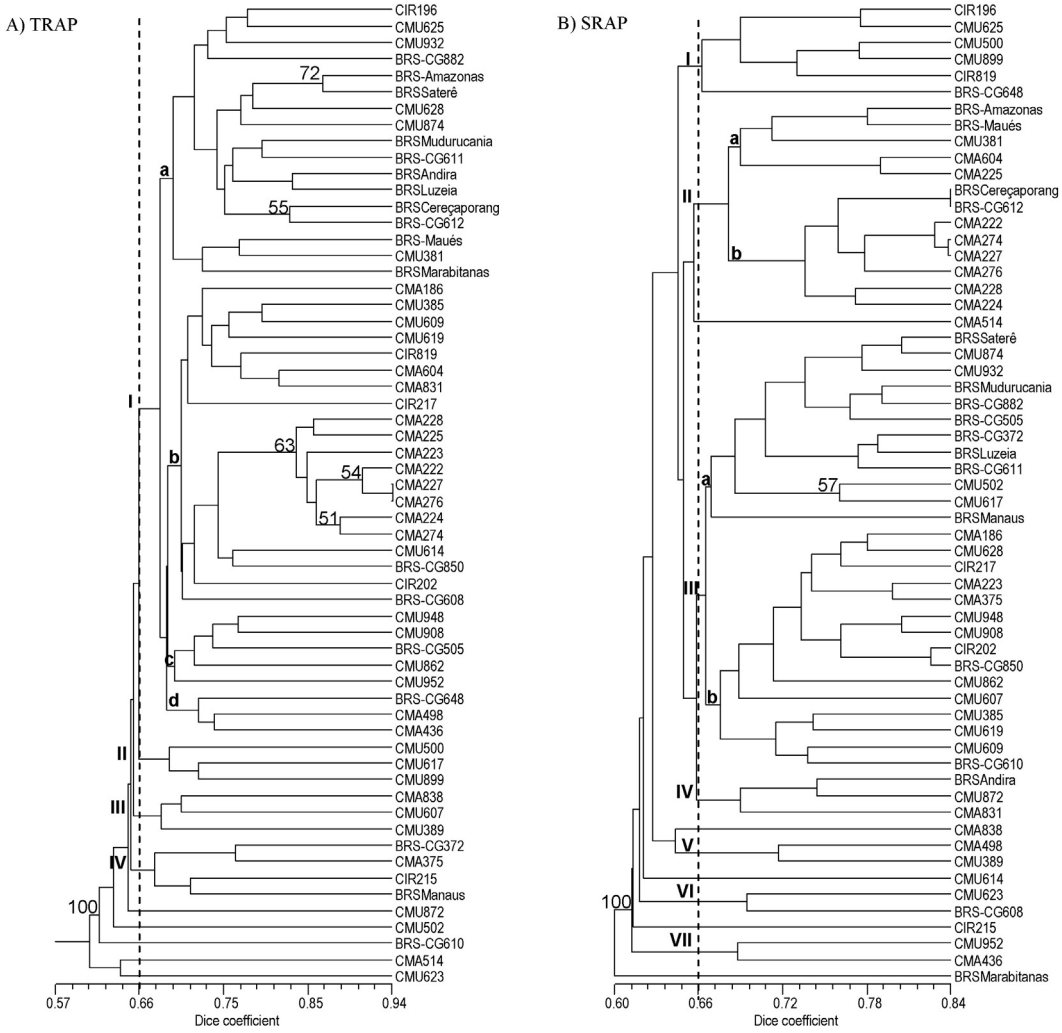

Fig. 1. Dendrogram generated by TRAP (A) and SRAP (B) markers, based on the UPGMA method, from 60 clonal guarana accessions. The numbers represent values from the bootstrap analysis.

ranged from 19 to 38, whereas the number of polymorphic bands ranged from 12 (*CystF + T03*) to 33 (*AuxR + T03*). The PIC values differed according to the primer combinations; the lowest value was 0.29 (*CystF + T03*), and the highest was 0.36 (*AuxR + T03*), with a mean value of 0.33 (Table 2).

3.2. SRAP marker

The eight primer pairs of the SRAP marker added up to 122 bands with 74.5% polymorphism. The level of polymorphism ranged from 66% (*Me15 + Em03*) to 82% (*Me04 + Em03*) for all primer combinations. The mean number of bands per combination was 15.25, and the total number of bands ranged from 19 (*Me10 + Em07*) to 10 (*Me01 + Em10*). The mean PIC was 0.35 among the SRAP marker combinations, but half of the combinations had a PIC of 0.37 (Table 2).

In the analysis of the dendrogram generated from the TRAP marker data, four main groups were identified, which were delimited by Dice similarity coefficients between 0.66 and 0.94 (Fig. 1-A). In the first group, 45 clones were distributed into four subgroups, and two of them were distinguished by the predominance of improved cultivars and similar clones. Eleven clonal cultivars were allocated to subgroup **Ia**, among which the pairs BRS-Amazonas/BRS-Saterê and BRS-Cereçaporanga/BRSCG-612 had a bootstrap value higher than 50. The largest subgroup (**Ib**) included 20 accessions from different germplasm types and origins, among which two clonal cultivars (BRSCG-850 and BRSCG-608) and a branch comprising the sample of similar phenotypes with a bootstrap value of 63. In the two smaller subgroups (**Ic** and **Id**), one comprised of the cultivar BRSCG-505 together with accessions from Maués (CMU) and another comprised of the cultivar BRSCG-648 together with accessions from Manaus (CMA).

The remaining groups were represented by one to three accessions more divergent relative to group **I**, among them the clonal cultivars BRS-Manaus and BRSCG-372 belonging to group **IV**. The BRSCG-610 cultivar was genetically more distant relative to the four groups identified together with clones CMA514 and CMU623, which had a maximal bootstrap value.

In the dendrogram built based on the SRAP marker, the guarana accessions were distributed into seven groups with a Dice similarity coefficient between 0.66 and 0.84. Group **I** was composed of five accessions from Iranduba (CIR) and Maués (CMU), associated with the clonal cultivar BRSCG-648. Group **II** was represented by accessions of different origins and similar clones separated into two subgroups; each subgroup contained a pair of more closely related clonal cultivars, BRS-Amazonas/BRS-Maués (**IIa**) and BRS-Cereçaporanga/BRSCG-612 (**IIb**). Group **III** concentrated 27 accessions in two subgroups differing in size and composition. The smallest subgroup (**IIIa**) combined eight cultivars (BRS-Saterê, BRS-Mudurucânia, BRSCG-882, BRSCG-605, BRSCG-372, BRSCG-Luzéia, BRSCG-611, BRS-Manaus) with three accessions originating from Maués (CMU). The largest subgroup (**IIIb**) was a mixture of accessions from different locations with two clonal cultivars, BRSCG-850 and BRSCG-610. Four small groups with two or less accessions were formed and two of them with one cultivar, BRS-Andirá (**IV**), and BRSCG-608 (**VI**). The BRS-Marabitanas cultivar was the most divergent relative to all the germplasm samples, with a maximal bootstrap value (Fig. 1-B).

The comparison of dendrograms (TRAP and SRAP) revealed a consistent level of genetic relationship among some genotypes, which persisted in the same group regardless of marker type. Six of out eight accessions with the same agronomic morphotype CMA (222, 224, 227, 228, 274, and 276) exhibited consistent genetic similarity, supported by bootstrap values above 50 for the TRAP marker. Cultivars BRS-Cereçaporanga and BRSCG-612, which differ in morpho-agronomical traits, were always grouped together, which was supported by the bootstrap value and the maximal similarity coefficient of 0.84 for the SRAP marker, indicating that they are the most closely related among the 18 cultivars evaluated. In all the guarana accessions analyzed, there were no pairwise genetic similarity values one, which ruled out the possibility of the occurrence of duplicates.

4. Discussion

The five TRAP primer combinations were more polymorphic than the eight for SRAP in the 60 guarana clones, which reflect the influence of the type of marker and variability captured in the sample. The percent polymorphism of the TRAP marker was compared with that found in other polyploids such as sugarcane (Devarumath et al., 2013) and wheat (Barakat et al., 2013). The polymorphism of the SRAP marker was similar to that obtained by 12 primer pairs in blackberry (Zhao et al., 2009) and lower than that obtained by 31 pairs in sugarcane (Suman et al., 2008).

The comparison between molecular markers has been used as a resource to access different genetic polymorphism sources. The dendrogram generated by the SRAP marker added three additional groups when the same cutoff value was adopted and distinguished two genotypes in the sample constituted by morpho-agronomically similar clones. The differences between the results are partially attributed to the features of each marker. SRAP amplifies preferentially intragenic fragments for polymorphism detection (Li and Quiros, 2001), whereas TRAP gathers information associated with expressed regions in the genome that are more conserved (Hu and Vick, 2003). The SRAP marker was also more informative in other comparisons such as with RAPD, Inter-Simple Sequence Repeat (ISSR) and SSR (Budak et al., 2004), Amplified Fragment Length Polymorphism (AFLP) (Ferriol et al., 2003), and EST-SSR (Huang et al., 2011).

Molecular markers have strengthened inferences on the genetic variation in cultivated and wild polyploid species. Studies using different nuclear markers have demonstrated a significant correlation of the ploidy levels with the number of alleles (Budak et al., 2005) and the band frequency (Gulsen et al., 2009). The microsatellite variation pattern has also provided support for the type of ploidy predominant in certain complex polyploids (Klie et al., 2014; Schie et al., 2014). More than five reproducible alleles in the same genotype were detected in the analysis of 59 alleles amplified by 10 EST-SSRs in 15 of the guarana cultivars used in this study, hampering conclusions regarding the number of copies or allele dosage (Angelo et al., 2014). In this study, the genetic variation detected by TRAP and SRAP markers was lower than expected given the level of complexity of the polyploid genotypes.

5. Conclusion

The TRAP and SRAP markers yielded complementary information on the genetic variability of the guarana germplasm. Therefore, combinations of the two markers have the potential to broaden the genetic characterization and assist in parental selection in the breeding program considering the complexity of polyploid genotyping.

Conflicts of interest

The authors have no conflicts of interest to declare.

Acknowledgments

Research supported by Embrapa (Brazilian Agricultural Research Corporation - grant number 0106010071006), National Counsel of Technological and Scientific Development (CNPq — Conselho Nacional de Desenvolvimento Científico e Tecnológico - grant number 556978/2009-9) and Amazon Research Foundation (FAPEAM — Fundação de Amparo à Pesquisa do Estado do Amazonas - grant number 062.01597/2014). This work is part of the first author's MS Dissertation (PPGBIOTEC/UFAM — Programa de Pós-Graduação em Biotecnologia at the Universidade Federal do Amazonas).

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