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Article

Authentication of Brazilian Ginseng using Bar-HRM analysis

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Hebanthe eriantha (Martius) Kuntze and *Pfaffia glomerata* (Spreng) Pedersen are medicinal plants popularly known as "Brazilian Ginseng" due to their similarity to *Panax ginseng*. In Brazil, they are sold as the same herb, despite their different pharmacological and toxicological properties. The morphological identification is difficult, which facilitates their adulteration. We report the application of the Barcode DNA High-Resolution Melting (Bar-HRM) using matK gene to differentiate both species in samples sold in the Brazilian market. Using the proposed method, we could discriminate and identify both species. Bar-HRM analysis allowed discriminating and identifying both species. It allowed the identification of *H. eriantha* and *P. glomerata* in 43.6% and 56.4% of the amplified samples, respectively. Of these, only seven samples were authenticated and, in 71.4% of the cases, adulterated. We concluded that Bar-HRM has proven to be a fast alternative method to authenticate plants under the common name "Brazilian Ginseng".

Keywords: Amaranthaceae. Eriantha. Glomerata. Hebanthe. Pfaffia.

INTRODUCTION

Hebanthe eriantha (Martius) Kuntze (formerly *Pfaffia paniculata*) and *Pfaffia glomerata* (Spreng) Pedersen are popularly known as "Brazilian Ginseng" (Oliveira, 1986; Rates, Gosman, 2002). These roots have been used for the treatment of physical fatigue, mental exhaustion, circulatory disorders; and due to their anti-inflammatory action (Oliveira, 1986; Rates, Gosmann, 2002; Costa *et al*., 2015). However, the properties of "Brazilian ginseng" can vary according to the species used. *Pfaffia glomerata* accelerates the healing of digestive ulcers (Freitas *et al*., 2004) and has a depressant effect on the nervous system (Fenner *et al*., 2008), contrary to the expected stimulating effect. *H. eriantha* has an antiproliferative activity in the hepatocarcinogenesis (da Silva *et al*., 2010) and a macrophage activity (Pinello *et al*., 2006).

Some studies on the medicinal use of *H. eriantha* and *P. glomerata* do not distinguish these plants, despite the presence of some chemicals that lead to differentiated pharmacological and toxicological properties. Their morphological identification is difficult due to their similarity, which can lead to the adulteration of products (Oliveira, 1986; Vigo *et al*., 2004). Besides, "Brazilian Ginseng" products are usually purchased, processed and unlabeled or unpackaged, which makes their identification even more challenging.

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Some studies have described chemical and molecular markers for these species individually, but none of them was used to identify both species (Flores *et al*., 2009; Figueira *et al*., 2011; Neves *et al*., 2016; Lian *et al*., 2019). These are the reasons why species identification methods using DNA, such as DNA barcoding, have been proposed and gradually included in the pharmaceutical compendia around the world (Sgamma *et al*., 2017).

Several DNA regions from the chloroplast genome have enough variation to be used as DNA barcodes to identify plant species (Hollingsworth, 2011). The maturase K (matK) gene is one of the high-level discriminatory regions (Yu *et al*., 2011). Analysis of this region is usually performed by sequencing method, which is expensive to be largely used. To overcome this problem, the High-Resolution Melting (HRM) has been recently proposed to exploit nucleotide polymorphisms as simple, cost-effective, and fast alternative. This analysis has already proven to be a reliable molecular approach for species identification and authentication (Osathanunkul *et al*., 2015; Costa *et al*., 2016; Osathanunkul, Madesis, 2019). Given this context, we aimed to evaluate if the Bar-HRM is useful for discriminating *H. eriantha* and *P. glomerata*.

MATERIAL AND METHODS

Plant materials

Six voucher specimes (three specimens of *P. glomerata* and three of *H. eriantha*) were morphologically identified by a specialist and used as reference species (Table I). In total, sixty commercial samples, sold as "Brazilian Ginseng," were bought in physical or online markets from several Brazilian cities (Table IS).

TABLE I - Reference samples of *Hebanthe eriantha* and *Pfaffia glomerata* used in this study

Taxon	Place of Collection	Voucher number
Hebanthe eriantha	Brazil/ São Paulo/ Campinas	CPQBA 50211, CPQBA 50212, CPQBA 50213
Pfaffia glomerata	Brazil/ São Paulo/ Campinas	CPQBA 2889, CPQBA 3833, CPQBA 4475

TABLE I - Reference samples of *Hebanthe eriantha* and *Pfaffia glomerata* used in this study

CPQBA: Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas from Universidade Estadual de Campinas (UNICAMP)

DNA extraction, amplification, and sequencing

DNA extraction was performed with using NucleoSpin® Plant II kit (Machereley-Nagel, Germany) following the manufacturer's protocol, using 30 mg of roots. DNA was amplified using matK-1KIM-F 5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3' and matK-3KIM-R 5'-CGTACAGTACTTTTGTG TTTACGAG-3' primers. PCR reactions was carried out with 1x PCR Buffer (Invitrogen), 1.5 nM of $MgCl_2$ (Invitrogen), 1,25pM of each primer, 0.25 mM of dNTP (Promega) and 1U of Platinum™ Taq DNA Polymerase (Invitrogen), 30 ng of DNA and ultrapure lab grade water up to final 12.25 µL. PCR was achieved under the following conditions: 94 ºC for 1 min, then 35 cycles of 95 ºC for 30 s, 52 ºC for 20 s, 72 ºC for 1min, with a final extension at 72 ºC for 5 min and 4 ºC. Cycle sequencing of both strands was conducted using BigDye® Terminator v3.1 (Thermo Fischer Scientific) in an ABI PRISM® 3500 Genetic Analyzer. Electropherograms were checked using BioEdit software v.7.0 (Hall, 1999). DNA sequencing results were compared with HRM analysis.

Bar-HRM design, analysis, and validation

Chloroplast matK gene sequence was generated from reference sequences of both species and aligned using BioEdit Sequence Alignment Editor version 7.2 (Hall, 1999). Primers were made manually by flanking polymorphic regions, and the annealing temperature and possible primer-dimer were checked using Perlprimer version 1.21 (Marshall, 2004). The uMelt-DNA Melting

version 2.0.2 (available at https://dna-utah.org/umelt/umelt.html) was used to predict the melting temperature of each amplicon.

Bar-HRM with pre-amplification was performed with a LightCycler® 96 (ROCHE) real-time PCR system. PCR amplifications were performed using EvaGreen® supermix SsoFast™ PCR, in a total volume of 10 µL, containing 5 µL of the supermix; 500nM of each primer, 5'-YTTCTTGAACGAATMYATTTCTAY-3'F and 5'-ACCTAACATAATGCRKGAAG-3'R (Table II); and 1 µL of ultrapure water to complete the final volume. Three concentrations of DNA amount (0.14, 1.4, and 14 ng) were tested for each one of the reference species to check the better condition of amplification, following the manufacturer's instructions. The concentration of 14 ng DNA was used for all run analysis.

TABLE II – matK partial gene sequences of *Pfaffia glomerata* and *Hebanthe eriantha* amplified by HRM primers in this study

Species	matK partial gene sequence	Tm	Amplicon size
Hebanthe eriantha	CTTCTTGAACGAATCCATTTCTACGGAAAGTTAAAATATCTAGTTAAAGTTA AGGCTTTTGCGGTTATCCTATGGTTTTTCAAAGAACCTTTCCCGCATTATGTTAGGT	75.5	109
Pfaffia glomerata	TTTCTTGAACGAATCCATTTCTACGTAAAGTTAAAATATCTAGTTAAAGTTA AGGCTTTTGGGGTTATCCTATGGTTTTTCAAAGAACCTTTCCCGCATTATGTTAGGT	75.0	109

The PCR temperature program included a denaturation step at 95°C for 2 min; followed by 40 cycles at 95°C for 30 s; 56°C for 30 s; and 72°C for 30 s. Melting analysis was performed at 95°C for 1 min, followed by 40°C for 1 min, then 65°C until 97°C, increasing at 0,07 °C/sec with 15 acquisitions of dye per grade. Reactions were performed in triplicate for each sample, including a negative control. Data were analyzed using LightCycler®96 SW v.1.1 (Roche Diagnostics, Risch-Rotkreuz, Switzerland). Genotypes were identified by examining normalized melting curves, difference, and derivative plots of the melting data. Melting temperature (Tm) data were statistically analyzed using Microsoft Excel 2010 to the calculation of standard deviation and confidence interval for each sample run in triplicate (Table IIS).

HRM results were compared with DNA sequencing results. The sensitivity (Altman, Bland, 1994) and specificity (Loong, 2003), precision (Lever *et al*., 2016) and likelihood ratio (Deeks, Altman, 2004) were evaluated.

RESULTS AND DISCUSSION

Bar-HRM performed with the specific matK primer successfully differentiated and identified both species commonly known as "Brazilian Ginseng". The melting profile allowed differentiation between *P. glomerata* and *H. eriantha* based on three SNPs, two transversions, T to G and G to C, and one transitions, T to C (Figure1).

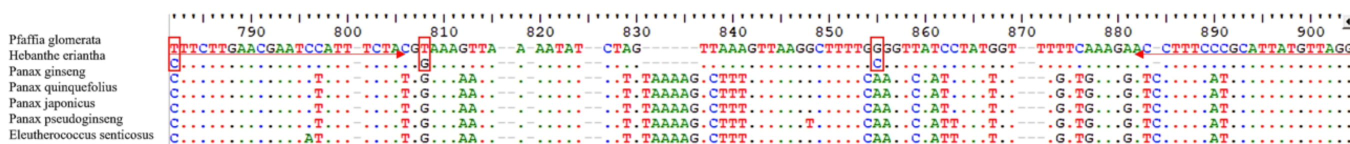
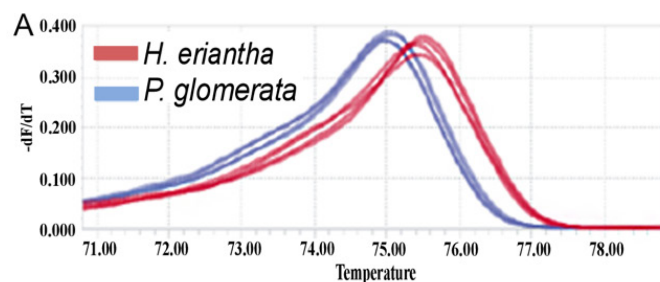


FIGURE 1 – Sequence alignment of the matK gene region showing nucleotide differences between species (red box) and primer design regions (red arrow).

Based on the conventional melting analysis, two groups of melt peaks could be observed: 75.0ºC and 75.5ºC for *P. glomerata* and *H. eriantha*, respectively (Figure 2A). The normalized melting curves from HRM analysis allowed the classification of the samples into two clusters, differentiating the species (Figure 2B).



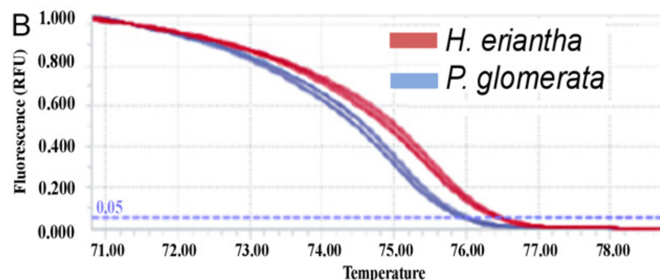


FIGURE 2 - A)Melting peaks of standard curves and B)normalized melting curves of Hebanthe eriantha and Pfaffia glomerata using Bar-HRM.

Our results from the Bar-HRM analysis were consistent when compared with DNA sequencing (Table IIS), as previously reported by Jilberto *et al.* (2017). The sensitivity of our reactions ranged between 92-93%, indicating a high rate of individuals identified by the proposed method. This means that of all samples analyzed, 92% of those identified by barcode sequencing as *P. glomerata* also amplified and belonged to the correct cluster when analyzed by the HRM method. And the same was true for 93% of the samples identified as *H. eriantha* by barcode sequencing.

The chosen primer also had 100% specificity for both species, showing that the test has a maximum performance to exclude the individuals of a particular species correctly. Due to the high specificity value found for the species, the positive likelihood ratio (LR+) was undefined, indicating a high probability that the individual belong to the species identified when the HRM analysis positively identifies a specific species. The values of the negative likelihood ratio (LR-) varied between 7-8%, indicating a high probability that the individual did not belong to the species when the HRM resulted in a negative value for a specific species. The accuracy was 100% for the HRM-matK primer, showing that the analysis did not have false positives.

In total, 43.6% of amplified samples were identified as *H. eriantha* and 56.4% as *P. glomerata* using Bar-HRM. Of these, only seven samples had the species listed on the label and, in 71.4% of the cases, were adulterated (they contained the wrong species).

Bar-HRM stands out as a fast process. After only 2 h of amplification and subsequent dissociation (about 2 minutes), the results are available. By promoting a DNA amplification of short duration, HRM analyzes small DNA fragments, which is beneficial for degraded material that is usually present in powder and dry plants (Kool *et al*., 2012; Särkinen *et al*., 2012). This method has also been applied with success to differentiate other plant species (Ganopoulos *et al*., 2012; Singtonat, Osathanunkul, 2015; Song *et al*., 2016).

We showed that the Bar-HRM was useful to identify both species, *H. eriantha* and *P. glomerata,* popularly grouped under the name "Brazilian Ginseng". Since this study is faster and cheaper than DNA sequencing, its use becomes easier in a wide variety of samples. HRM may be included as a quality control protocol for raw materials of plants and promote consumer confidence. However, its use still requires regulation in each country.

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TABLE IS - Commercial samples sold as "Brazilian Ginseng" used in this study

ID	Commercial name (label)	Lot	Place of purchase	Type	Plant part
01	*Pfaffia* (Brazilian ginseng)	12015	Internet	Powdered	X
02	Ginseng (*Pfafia paniculata*)	52468	Internet	Powdered	X
03	Brazilian ginseng	110/14	Internet	Powdered	X
04	Ginseng	X	Casa das Ervas - Vila Rubim - ES	Capsule	X
05	Ginseng	X	Natura Minas – Vila Velha - ES	Fragment	Leaf and flowers
06	Ginseng (*Pfafia paniculata*)	2	Mundo Verde – Vila Velha - ES	Powdered	Root
07	Ginseng	X	PequenaSela – Vila Rubim - ES	Powdered	X
08	Ginseng	X	Mercearia Rodrigues – Vila Rubim - ES	Powdered	X
09	Ginseng (*Pfafia paniculata*)	FAFI001	Natura Minas – Vila Velha - ES	Powdered	Root
10	Ginseng	X	PharmaErvas – Vila Rubim - ES	Powdered	X
11	Ginseng	X	Guarapari - ES	Powdered	X
12	Ginseng	X	Araraquara - SP	Powdered	X
13	Ginseng	X	Manaus - AM	Powdered	X
14	Ginseng (*Pfaffia* ssp)	32	Rio de Janeiro - RJ	Fragment	Root
15	Ginseng	X	Rio de Janeiro - RJ	Fragment	Root
16	Ginseng (*Pfafia paniculata*)	X	Rio de Janeiro - RJ	Powdered	X
17	Ginseng Powdered	X	Barra da Tijuca - RJ	Powdered	X
18	Ginseng Powdered	X	Rio de Janeiro - RJ	Powdered	X
19	Ginseng	1220	Ribeirão Preto - SP	Powdered	X
20	Ginseng	X	Ribeirão Preto - SP	Fragment	Root
21	National ginseng (*Pfaffia paniculata*)	1224	Ribeirão Preto - SP	Powdered	X
22	Ginseng	X	Ribeirão Preto - SP	Powdered	X

TABLE IS - Commercial samples sold as "Brazilian Ginseng" used in this study

ID	Commercial name (label)	Lot	Place of purchase	Type	Plant part
23	Pfaffia (*Pfaffia glomerata*)	2581	Ribeirão Preto - SP	Powdered	X
24	Ginseng	X	Belo Horizonte - MG	Capsule	X
25	Ginseng	X	Belo Horizonte - MG	Capsule	X
26	Ginseng	X	Belo Horizonte - MG	Capsule	X
27	Ginseng	X	Belo Horizonte - MG	Powdered	X
28	Ginseng	X	Belo Horizonte - MG	Powdered	X
29	Brazilian ginseng (*Pfaffia paniculata*)	X	Belo Horizonte - MG	Powdered	X
30	Ginseng	X	Belo Horizonte - MG	Powdered	X
31	Ginseng	X	Belo Horizonte - MG	Powdered	X
32	Ginseng	X	Belo Horizonte - MG	Powdered	X
33	Brazilian ginseng (*Pfaffia paniculata* Mart)		Belo Horizonte - MG	Powdered	Root
34	Ginseng	X	Belo Horizonte - MG	Powdered	X
35	Ginseng	X	Belo Horizonte - MG	Powdered	X
36	Ginseng (Pfaffia)	X	Belo Horizonte - MG	Powdered	X
37	Ginseng	X	Belo Horizonte - MG	Powdered	X
38	Brazilian Ginseng (*Pfaffia paniculata*)	X	Belo Horizonte - MG	Powdered	X
39	Korean Ginseng Tea gold	X	Belo Horizonte - MG	Powdered	X
40	Ginseng	X	Belo Horizonte - MG	Powdered	X
41	Ginseng	X	Belo Horizonte - MG	Fragment	Leaf and stem
42	Ginseng	X	Belo Horizonte - MG	Powdered	X
43	Ginseng	X	Belo Horizonte - MG	Powdered	X
44	Ginseng	X	Casas das Ervas - Vila Rubim - ES	Powdered	X
45	Ginseng	X	Belo Horizonte - MG	Powdered	X
46	Ginseng	X	Guaçuí - ES	Powdered	X
47	Ginseng (*Pfaffia paniculata*)	X	Guarapari - ES	Powdered	X
48	Ginseng Powdered	X	São Paulo - SP	Powdered	X
49	Ginseng Powdered	X	São Paulo - SP	Powdered	X
50	Ginseng Powdered	X	São Paulo - SP	Powdered	X
51	Several	X	São Paulo - SP	Powdered	X

TABLE IS - Commercial samples sold as "Brazilian Ginseng" used in this study

ID	Commercial name (label)	Lot	Place of purchase	Type	Plant part
52	Ginseng Powdered KG	X	São Paulo - SP	Powdered	X
53	Ginseng Powdered	X	São Paulo - SP	Powdered	X
54	GingSeng	X	São Paulo - SP	Powdered	X
55	Ginseng root	X	São Paulo - SP	Powdered	X
56	Ginseng powdered 12	X	São Paulo - SP	Powdered	X
57	Ginseng powdered	X	São Paulo - SP	Powdered	X
58	Ginseng powdered	X	São Paulo - SP	Powdered	X
59	Ginseng - 209	X	São Paulo - SP	Powdered	X
60	Ginseng bulk	X	São Paulo - SP	Powdered	X

Note: ID: sample identification number. X: information not provided

TABLE IIS - Application of the developed method on commercial samples

Sample code	Real Time PCR			DNA barcoding
	SD	Confidence (95%)	HRM	
1	0,05	74,56±0,12	*P. glomerata*	*P. glomerata*
2	-	-	Not amplified	Not amplified
3	-	-	Not amplified	Not amplified
5	0,04	75,45±0,09	*H. eriantha*	*H. eriantha*
6	0,04	74,60±0,10	*P. glomerata*	*P. glomerata*
7	0,02	74,64±0,06	*P. glomerata*	*P. glomerata*
8	0,03	75,29±0,07	NRC	*H. eriantha* *
10	0,04	75,39±0,11	*H. eriantha*	*H. eriantha*
11	-	-	Not amplified	NRC
12	0,12	74,77±0,29	*P.glomerata*	*P. glomerata*
13	0,10	75,44±0,25	*H. eriantha*	*H. eriantha*
14	0,02	75,49±0,05	*H. eriantha*	*H. eriantha*
15	0,12	75,35±0,15	*H. eriantha*	*H. eriantha*
16	0,21	74,96±0,52	*P. glomerata*	*P. glomerata*
17	-	-	Not amplified	*P. glomerata**
18	0,26	74,87±0,64	*P. glomerata*	*P. glomerata*
19	0,01	74,90±0,02	*P. glomerata*	*P. glomerata*

TABLE IIS - Application of the developed method on commercial samples

Sample code	Real Time PCR			DNA barcoding
	SD	Confidence (95%)	HRM	
20	0,04	74,71±0,09	*P. glomerata*	*P. glomerata*
21	-	-	Not amplified	NRC
22	0,20	74,97±0,49	*P. glomerata*	*P. glomerata*
23	0,04	75,36±0,08	*H. eriantha*	*H. eriantha*
24	0,23	75,36±0,25	*H. eriantha*	*H. eriantha*
25	0,09	74,80±0,23	*P. glomerata*	*P. glomerata*
26	0,04	75,39±0,12	*H. eriantha*	*H. eriantha*
27	0,09	75,35±0,24	*H. eriantha*	*H. eriantha* *
28	0,04	74,61±0,10	*P. glomerata*	*P. glomerata*
30	-	-	Not amplified	*P. glomerata**
31	0,16	74,79±0,39	*P. glomerata*	*P. glomerata*
32	0,07	74,64±0,17	*P. glomerata*	*P. glomerata*
33	0,14	74,76±0,35	*P. glomerata*	*P. glomerata*
34	0,08	74,72±0,21	*P. glomerata*	*P. glomerata*
35	-	-	Not amplified	*P. glomerata**
36	0,02	74,73±0,04	*P. glomerata*	*P. glomerata*
37	-	-	Not amplified	NRC
38	0,29	75,02±0,72	*P. glomerata*	*P. glomerata*
39	0,26	74,87±0,64	*P. glomerata*	*P. glomerata*
40	-	-	Not amplified	*P. glomerata*
41	0,13	74,73±0,34	*P. glomerata*	*P. glomerata*
42	0,02	75,37±0,05	*H. eriantha*	Not amplified
43	0,07	74,66±0,18	*P. glomerata*	*P. glomerata*
44	0,13	74,74±0,34	*P. glomerata*	*P. glomerata*
45	-	-	Not amplified	NRC
46	0,04	75,36±0,11	*H. eriantha*	*H. eriantha*
47	-	-	Not amplified	Not amplified
48	-	-	Not amplified	Not amplified
49	0,01	75,31±0,02	NRC	*H. eriantha* *
50	-	-	Not amplified	NRC
51	0,25	75,25±0,62	*H. eriantha*	*H. eriantha*
52	0,14	75,41±0,05	*H. eriantha*	*H. eriantha*

TABLE IIS - Application of the developed method on commercial samples

Sample code	Real Time PCR			DNA barcoding
	SD	Confidence (95%)	HRM	
53	0,02	74,92±0,04	*P. glomerata*	*P. glomerata*
54	0,06	75,37±0,15	*H. eriantha*	*H. eriantha*
55	-	-	Not amplified	NRC
56	0,05	75,43±0,13	*H. eriantha*	*H. eriantha*
57	0,01	75,31±0,02	NRC	NRC
58	0,03	75,42±0,08	*H. eriantha*	NRC
59	-	-	Not amplified	NRC
60	-	-	Not amplified	NRC
61	-	-	Not amplified	NRC
62	-	-	Not amplified	*P.glomerata*
65	0,05	75,56±0,13	*H. eriantha*	*H. eriantha*

Note: *Sample with mixture of other unrelated species. NRC (No Reference Cluster).

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