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# Molecular authentication of *Maytenus* sp by PCR-RFLP

# Autenticação molecular de *Maytenus* sp por PCR-RFLP

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## Abstract

*Maytenus aquifolia* and *Maytenus ilicifolia* are native plants from South America and popularly known as 'Espinheira-santa'. Both are used as tea due to their efficiency in the treatment of ulcer, gastritis and indigestion. However, adulteration of processed *Maytenus* genus tea with *Sorocea* genus may happen due to their botanical similarity, compromising the quality of the products and opening a derogatory business opportunity that may lead to the discrediting of medicinal plant products. This study aimed to distinguish *Maytenus* sp and *Sorocea bonplandii* by PCR-RFLP of a chloroplast DNA (cpDNA) intergenic region. Three commercial products of processed tea leaves of *Maytenus* sp, and *in natura* leaves of *Maytenus* sp and *S. bonplandii* were analyzed. PCR detected unique fragments for all samples *in natura*. The *trnH-psbA* region amplicon of both *M. ilicifolia* and *M. aquifolia* was 660 bp, and for *S. bonplandii* was 565 bp. These PCR products can be used as markers to distinguish the two genera. Forty-five percent of the processed samples presented only *Maytenus* genus, without adulterations. However, the amplification of 38% of the samples suggests adulteration with *S. bonplandii* while 17% seem to be adulterated with another plant (fragment of 649 bp in brand A and 690 bp in brand B). Three out of the fifteen restriction enzymes were able to detect *M. ilicifolia* and *M. aquifolia in natura* and in processed leaf samples. It was concluded that PCR technique is efficient to distinguish *Maytenus* sp from *S. bonplandii*, and other adulterating plants in processed commercial products of 'Espinheira-santa' tea. The *trnH-psbA* spacer of cpDNA is easily amplified and has satisfactory discriminating capacity to help in authentication processes of samples of the genera *in natura* and in processed plants.

**Key words:** *Maytenus aquifolia, Maytenus ilicifolia, Sorocea bonplandii,* Celastraceae*,* Moraceae*, trnH-psbA*

## Resumo

*Maytenus aquifolia* e *Maytenus ilicifolia* são plantas nativas da América do Sul e conhecidas popularmente como "Espinheira-santa". Ambas são usadas como chá devido à sua eficiência no tratamento de gastrite, úlcera e indigestão. No entanto adulteração de chá do gênero *Maytenus* com o gênero *Sorocea* pode acontecer devido a sua semelhança botânica e isto compromete a qualidade dos produtos, abrindo uma oportunidade para comércio depreciativo que leva ao descrédito das plantas medicinais. Este estudo teve como objetivo distinguir *Maytenus* sp e *Sorocea bonplandii* por PCR-RFLP de DNA da região intergênica do cloroplasto (cpDNA). Três produtos comerciais de chá de *Maytenus* sp foram analisados, e a análise foi feita também em folhas *in natura* de *Maytenus* sp e de *S. bonplandii*. PCR detectou fragmentos únicos para todas as amostras *in natura*. O amplicon da região *trnH-psbA* de *M. ilicifolia* e *M. aquifolia* foi de 660 pb e para *S. bonplandii* foi de 565 pb. Os produtos de PCR podem ser usados

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como marcadores para distinguir os dois gêneros. Quarenta e cinco por cento das amostras processadas apresentaram apenas o gênero *Maytenus*, sem adulterações. No entanto, a amplificação de 38% das amostras sugere adulteração com *S. bonplandii*, enquanto 17% parecem terem sido adulteradas com uma outra planta (fragmento de 649 pb na marca A e 690 pb na marca B). Três das quinze enzimas de restrição foram capazes de detectar *M. ilicifolia* e *M. aquifolia in natura* e em amostras de folhas processadas. Concluiu-se que a técnica de PCR é eficiente para distinguir *Maytenus* sp de *S. bonplandii* e outras plantas adulterantes nos produtos comerciais de chá de "Espinheira-santa". O espaçador *trnH-psbA* de cpDNA é facilmente amplificado e tem capacidade discriminante satisfatória para ajudar em processos de autenticação dos gêneros em amostras *in natura* e processadas de plantas.
**Palavras-chave:** *Maytenus aquifolia, Maytenus ilicifolia, Sorocea bonplandii,* Celastraceae*,* Moraceae*, trnH-psbA*

## Introduction

*Maytenus aquifolia* Mart. and *Maytenus ilicifolia* Mart. ex. Reissek (Celastraceae) are native plants of South America and popularly known as 'Espinheira-santa' (JORGE et al., 2004). Both are used as tea due to their efficiency in the treatment of ulcer, gastritis and indigestion (CARLINI; BRAZ, 1988). The high demand for these medicinal plants has increased their uncontrolled extraction in their natural environment. Although they are well known as *Maytenus* sp, they are morphologically confused with *Sorocea bonplandii* (Baill.) W.C.Burger, Lanj. & Boer (Moraceae), which is also a native plant from the same environment. The adulteration of processed *Maytenus* tea with *Sorocea* genus apparently does not harm the consumers' health because *S. bonplandii* has analgesic and antiulcerogenic properties (GONZALEZ et al., 2001; VILEGAS et al., 1998). However, the presence of *S. bonplandii* in 'Espinheira-santa' teas indicates the need of authentication of medicinal plants used in commercial products.

Authentication of medicinal plants by taxonomists or by chemical markers is limited due to the specific knowledge required from the former (NODARI; GUERRA, 1999; SONG et al., 2009) and the lack of trust in the latter that are susceptible to environmental changes (UM et al., 2001; ZHAO et al., 2007). The use of DNA markers in the authentication process can solve some of these problems, as DNA is not susceptible to alteration due to cultivation conditions and can be obtained from a small amount of biological material, even after processed (INFANTE et al., 2006; UJIHARA et al., 2009).

In plants, in general, the mitochondrial genome presents low variability and its utility as molecular marker for identification or authentication of plants products is low (CHASE et al., 2007). However, intergenic regions of chloroplast DNA (cpDNA) have been used successfully to characterize angiosperms (FAZEKAS et al., 2008; HOLLINGSWORTH et al., 2009; LAHAYE et al., 2008; SONG et al., 2009) and it also could be a tool to distinguish *Maytenus* and *Sorocea* genera. Moreover, there are no data in literature about the use of DNA markers to distinguish these genera. Thus, due to the absence of studies on the authentication of *Maytenus* sp by DNA sequences and the importance of the authenticity of plants used as medicine to the sustainable development of the natural product industry, this study aimed to distinguish *Maytenus* sp from *S. bonplandii* using the *trnH-psbA* intergenic regions of cpDNA by PCR-RFLP technique to identify a molecular marker between *in natura* as well as commercially processed *Maytenus* sp and *S. bonplandii*.

## Material and Methods

Leaves from six *M. aquifolia* (Ma), eight *M. ilicifolia* (Mi) and seven *S. bonplandii* (Sb) bushes were harvested at the Medicinal Plant Herbarium of the Universidade Paranaense in Umuarama-

PR, Brazil. The plants were identified and a voucher specimen of each species was deposited at the Educational Herbarium of the Universidade Paranaense (registration numbers 2063, 2064 and 2065). Each leaf was washed with ethanol 70% and distilled water and stored at –70 ºC.

Some dried leaf teas of 'Espinheira-santa' (Ma or Mi) were purchased from local traders. They were from three different broadly-commercialized brands that were identified as A, B or C, and four packages of each of them were chosen randomly and coded as 1, 2, 3 or 4. The total content of each package was washed twice with sodium hypochlorite 1% for 5 min under agitation and then washed with ultra-pure water for 5 min. Next, they were dried at 60 ºC in an air circulation oven for 90 min. After that, it was ground in an analytical grinder, sieved (1.0 mm mesh) and stored in glass flasks with silica gel at room temperature (25 ºC) in the dark. Each package of processed leaves was analyzed in triplicate and codified as sample I, II or III.

In order to extract genomic DNA (DOYLE; DOYLE, 1987), 4 $cm^2$ of a leaf *in natura* was used (Ma, Mi or Sb), stored in micro tubes and frozen again for 20 min at –70 ºC. Then, the material was macerated and extraction buffer was added. The resulting DNA was suspended in TE (1 mM Tris-HCl; 0.1 mM EDTA pH 8.0) and kept frozen at –20 ºC. In the analysis of the commercial samples, 5 mg of ground leaves were used according to the previous protocol (DOYLE; DOYLE, 1987) without freezing and maceration. The DNA extractions were done in triplicate, and the DNA concentration and purity degree of each sample were determined by spectrophotometry.

Universal primers described by Cuénoud et al. (2002) (Table 3) were used to amplify the *trnH-psbA* intergenic spacer regions of cpDNA. Amplification process occurred in a thermo cycler (Mastercycler Gradiente – Eppendorf) programmed for initial denaturation at 94 ºC for 4 min; followed by 35 cycles: denaturation at 94 ºC for 30 s, annealing at 60 ºC for 30 s and extension at 72 ºC for 30 s, followed by final extension at 72 ºC for 7 min. The final 25 µL volume reaction contained DNA (25 ng), $MgCl_2$ (1.5 mM), enzyme buffer (200 mM Tris-HCl pH 8.4; 500 mM KCl), dNTP (100 µM of each), primer (0.2 µM), and *Taq* DNA polymerase (1.5 U). The amplification products were submitted to electrophoresis in agarose gel at 1% with ethidium bromide and photographed under ultraviolet light. The amplification reaction was replicated three times.

**Table 1.** Sequence of primers used in the amplification of *trnH-psbA* intergenic spacer of chloroplast DNA of *Maytenus* sp and *Sorocea bonplandii*.

| Primer | Sequence (5' → 3') |
|---|---|
| *trnHf* | CGCGCATGGTGGATTCACAATCC |
| *psbA3F* | GTTATGCATGAACGTAATGCTC |

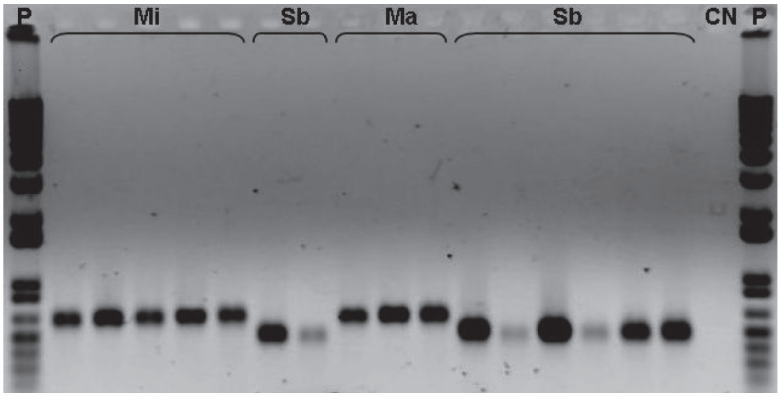
**Source:** Elaboration of the authors.

For leaves *in natura*, PCR products were digested with 15 restriction endonucleases (*Acc*I, *Alu*I *Bam*HI, *Eco*RI, *Hae*III, *Hind*III, *Hinf*I, *Hha*I, *Kpn*I, *Mbo*I, *Mse*I, *Rsa*I, *Sal*I, *Pst*I, *Pvu*II) following the manufacturer's recommendation. The fragments were separated by electrophoresis in agarose gel at 1.5% with ethidium bromide and photographed under ultraviolet light. The fragment size of PCR products before and after the digestion was estimated in base pairs (bp) by comparing them to 1 kb plus DNA ladder®. Only the enzymes that could cut the amplicons of leaves *in natura* were used for the amplicons of processed leaves.

## Results and Discussion

The *trnH-psbA* intergenic spacer presented enough variation to distinguish the analyzed genera in this study. In Fig. 1, it can be observed that *M. ilicifolia* and *M. aquifolia* amplifications generated fragments with 660 bp, and for *S. bonplandii* with 565 bp. In literature, different applications of PCR and PCR-RFLP are reported, using nuclear or cpDNA regions in order to distinguish ginseng species (*Panax ginseng* and *Panax quinquefolius*) (NGAN et al., 1999) and medicinal species of *Hedyotis diffusa* and *Hedyotis corymbos* (LI et al., 2010), in the authentication of the Polygonaceae family in Chinese pharmacopoeia (SONG et al., 2009) and in *Actinidia macrosperma* (ZHAO et al., 2007). Some of these studies did not obtain results only with PCR, making the PCR-RFLP analysis necessary.

**Figure 1.** Amplification products of *trnh-psbA* intergenic spacer of chloroplast DNA from leaves *in natura* of *Maytenus ilicifolia* (Mi), *Maytenus aquifolia* (Ma) and So*rocea bonplandii* (Sb). NC = Negative Control and P = 1 kb plus DNA ladder.




**Source:** Elaboration of the authors.

Another aspect that reinforces the trustworthiness of the use of this technique to distinguish *Maytenus* genus from *S. bonplandii* is the high annealing temperature during amplification (60 ºC) that guarantees a very specific amplification. Even with the high temperature used during primer annealing, the reactions generated a great number of copies of the target sequence. Thus, it was observed that size polymorphism obtained only with PCR allowed distinguishing *Maytenus* genus of *S. bonplandii*. These results corroborate the high discriminating power of *trnH-psbA* cpDNA region, suggesting the possibility of adopting this segment as a marker to distinguish *Maytenus* genus from its main adulterant, *S. bonplandii,* with the advantage of not using DNA sequencing.

In the analysis of processed leaves (tea) of the *Maytenus* genus, 20% of the samples showed no amplification of *trnH-psbA* (Table 1). The absence of amplification suggests that the DNA was degraded during leaf processing once DNA from *in natura* leaves of *M. ilicifolia*, *M. aquifolia* and *S. bonplandii* were able to anneal even at specific high amplification temperature (60 °C).

**Table 2.** Amplification result of the *trnH-psbA* region of chloroplast DNA from processed leaves of commercial brands of *M. ilicifolia* and *M. aquifolia* known as 'Espinheira-santa', purchased in Umuarama-PR.

| Processed leaves | | | *Maytenus* genus | *Sorocea bonplandii* | Another genus |
|---|---|---|---|---|---|
| A | 1 | I | - | - | + |
| | | II | - | - | + |
| | | III | - | - | + |
| | 2 | I | + | - | - |
| | | II | + | - | - |
| | | III | + | - | - |
| | 3 | I | + | - | - |
| | | II | + | - | - |
| | | III | + | - | - |
| | 4 | I | + | - | - |
| | | II | + | - | - |
| | | III | + | - | - |
| B | 1 | I | - | - | + |
| | | II | - | - | + |
| | | III | aa | aa | aa |
| | 2 | I | + | - | - |
| | | II | aa | aa | aa |
| | | III | aa | aa | aa |
| | 3 | I | aa | aa | aa |
| | | II | aa | aa | aa |
| | | III | aa | aa | aa |
| | 4 | I | + | - | - |
| | | II | + | - | - |
| | | III | + | - | - |
| C | 1 | I | + | + | - |
| | | II | + | + | - |
| | | III | + | + | - |
| | 2 | I | - | + | - |
| | | II | - | + | - |
| | | III | - | + | - |
| | 3 | I | - | + | - |
| | | II | - | + | - |
| | | III | aa | aa | aa |
| | 4 | I | - | + | - |
| | | II | - | + | - |
| | | III | - | + | - |

**Legend**: A, B and C = brand; 1, 2, 3 and 4 = processed unit (package); I, II, III = replication of the package analysis. + = positive result; – = negative result; aa = absence of amplification.
**Source:** Elaboration of the authors.

Only 45% of the processed leaves showed *Maytenus* genus without adulterants. Adulteration with *S. bonplandii* was detected in 38% of the samples and 17% of the processed leaves were mixed with another type of plant (fragments with 649 bp in brand A and 690 bp in brand B). Thus, 55% of the amplified processed leaves presented some kind of plant adulterant. Tea adulteration with *S. bonplandii* as well as other plants suggests problems during the processing and quality control of *Maytenus* genus teas.

Even though there was enough polymorphism to distinguish *Maytenus* genus and *S. bonplandii* by PCR, 15 restriction endonucleases were utilized in the restriction analysis of the *trnH-psbA* region of *in natura* leaves, which results on additional information to distinguish both genera. Among the tested enzymes, *Mbo*I, *Mse*I and *Rsa*I were informative, detecting polymorphism in the *trnH-psbA* region (Table 3).

**Table 3.** Estimated size (bp) of fragments obtained by digestion with restriction endonucleases of the *trnH-psbA* intergenic spacer of chloroplast DNA from *M. ilicifolia, M. aquifolia* and *S. bonplandii* leaves *in natura*.

| | ***M. aquifolia* and *M. ilicifolia*** | | ***S. bonplandii*** | |
|---|---|---|---|---|
| **Enzyme** | **Fragments (bp)** | **Total of fragments (bp)** | **Fragments (bp)** | **Total of fragments (bp)** |
| ***Mse* I** | 256; 102 | 358 | 213; 115 | 328 |
| ***Mbo* I** | 350; 162 | 512 | 565 | 565 |
| ***Rsa* I** | 375; 208; 100 | 683 | 565 | 565 |

**Source:** Elaboration of the authors.

*Mse*I produced restriction fragments for *Maytenus* genus and *S. bonplandii*. *Mbo*I and *R*sal generated fragments for *Maytenus* sp, but they did not recognize restriction sites for *S. bonplandii* (Table 2). Regardless the number of tested enzymes, it was not possible to differ *M. ilicifolia* from *M. aquifolia*. Thus, these enzymes are additional markers for identification of *Maytenus* genus and *S. bonplandii* by PCR-RFLP. The amplicons of processed leaves digested with *Mbo*I, *Mse*I and *Rsa*I presented results that corroborated the ones found in *in natura* leaves.

It was concluded that the PCR technique is efficient to distinguish *Maytenus* sp from *S. bonplandii*, and detecting the presence of other adulterating plants. However, the PCR-RFLP technique is inefficient to distinguish the species that are very close like *M. ilicifolia* and *M. aquifolia*. The *trnH-psbA* spacer of cpDNA is easily amplified and has satisfactory discriminating capacity to authenticate genera of *in natura* and in processed plants.

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