Forensic Botany and Forensic Chemistry working together: advances on applications of plant DNA barcoding in complementing some specific demands of forensic sciences in Brazil – A case study.

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Abstract

Background: Forensic Chemistry is a popular and widely accepted discipline for the assessment of suspected plant material. Joining reliability, speed of analysis, cost-effectiveness and relatively effortless bench work, it is a gold standard for evaluating plant material suspected to be an illicit drug. Notwithstanding, sometimes it can be a limited technique by the low levels or absence of chemicals upon which analysis is based, *e.g.*, in cases involving seeds or tiny amounts of material. This is the present case: illicit drugs or controlled chemicals were not found in the suspicious plant material. Once remained aforementioned suspicion, we had to appeal, therefore, to the plant DNA barcoding technique. **Results**: Using multiple sets of primers (7 pairs), barcodes were obtained from 10 specimens, representing 5 *taxa* from 2 packets containing botanical material, homogenous at first sight. Most of the specimens (4 samples) were from the same nature, which we could identify at the species level (*Artemisia absinthium*). **Significance**: At this degree of complexity, it is the first report about an effective effort using an association between Forensic Botany and Forensic Chemistry techniques in order to assess the real nature of a suspected plant material in Brazil. Such approach was done mainly due the claims from the national judiciary system. Notwithstanding the rigor ever and routinely implemented in forensic sciences, sometimes it is imperative to lay hand on techniques not routinely employed in order to satisfy the final client on its questions. This case, thus, can serve as salutary self-criticism about the frequent tendency of least effort to analysis. The combination of DNA barcoding with Forensic Chemistry techniques, therefore, can offer an efficient solution to evaluate plant material adequately from now on.

Introduction

Living plants and dried vegetable matter are some of frequently seized items suspected of being or to contain illicit drugs/controlled chemicals. Analysis of these items are routinely performed through techniques which, altogether, are termed Forensic Chemistry[1].

There are, although, many situations where Forensic Chemistry is, somehow, limited and macroscopic and microscopic examinations may fail.

In this context, plant DNA barcoding has emerged as a powerful tool on routine applications in forensics. This technique is reliable and is not affected by external factors such as climate, age or plant part, so that DNA barcoding methods have also been recently applied even to the authentication of herbal drug materials for industrial quality assurance[2].

Some studies suggested that the DNA barcoding should be used in a complementary manner with chemical analyses for species identification in herbals[3]. Here we describe a case where it was pivotal. Taking into account all the advantages and inherent limitations of spectroscopic methods and DNA barcoding, the combination of the two techniques could be a synergistic effort for comprehensive assessment of vegetable matters.

Material and Methods

In 2013, packets containing botanical material, homogenous at first sight, were seized while they were mailed. When suspect packets (relating to illicit drugs) are detected, they are usually send to Brazilian Federal Police, where standard analysis is based on chemical tests. Until 2015 that vegetable material was submitted and retested basically through spectroscopic methods - Gas Chromatograph coupled to a Mass Spectrometer (GC-MS) and Fourier Transform Infrared Spectrometer (FTIR).

In 2016, the forensic genetics laboratory received that material. It consisted of two packets containing botanical material formed by fragmented or smashed leaves, flowers, seeds and stems. Ten samples (Figure 1) were taken and grinded in a proper mill (MM400 Retsch®). Total DNA from each plant tissue fragment was extracted with PrepFiler Express BTA™ Forensic DNA Extraction employing an AutoMate Express™ DNA Extraction System robotic workstation (Thermo Fisher Scientific Inc.).

Seven universal PCR primers were used to amplify five different land plant DNA regions[4-9] - matK, rbcL, trnL/IGS – intergenic spacer_trnL-trnF, ycf1, ITS1/ITS2. PCR conditions, depending on primer, and sequencing procedures were performed as recommended in aforementioned literature adapted in our laboratory. Consensus sequences yielded were then compared to those deposited on data banks (Genbank[10] and BOLD systems V3[11]).

Employing the most similar sequences found on data banks, phylogenetic trees were constructed with software MEGA 6[12] using the neighbor-joining method with bootstrap support values calculated by running at least 1000 replicates.

In order to identify the nature of this questioned material, we took into account Genbank and BOLD systems V3 similarity results, the representativeness of the plant groups in the databases and, principally, the existence of phylogenetic studies performed previously for the plant groups in focus.

Figure 1. Example of four samples (out of ten), which are probably associated to the genus *Elymus* spp. (2061Q9), *Sambucus* spp. (2061Q10), *Cynodon* spp. (2061Q11) and, certainly, to the species *Artemisia absinthium* (2061Q12).

Results

Chemical analysis found *thujone* in the botanical material. It is a chemical compound naturally encountered in many plant species, notoriously *Artemisia absinthium*[13].

Genetic analysis yielded 30 good quality sequences from the 10 samples throughout the five regions analyzed. All sequences resulted in high levels of genetic similarity once searched in data banks.

Six samples reached highest genetic similarity to genera *Sambucus* spp., *Clematis* spp., *Elymus* spp. and *Cynodon* spp., above the 99% threshold. Four samples showed a similarity range between 98 and 100% to the species *Artemisia absinthium*. Based on the representativeness of the groups in the consulted databases and the existence of previous phylogenetic studies, we were able to construct phylogenetic trees and to achieve secure conclusions only about the identity of the later four samples (Figure 2).

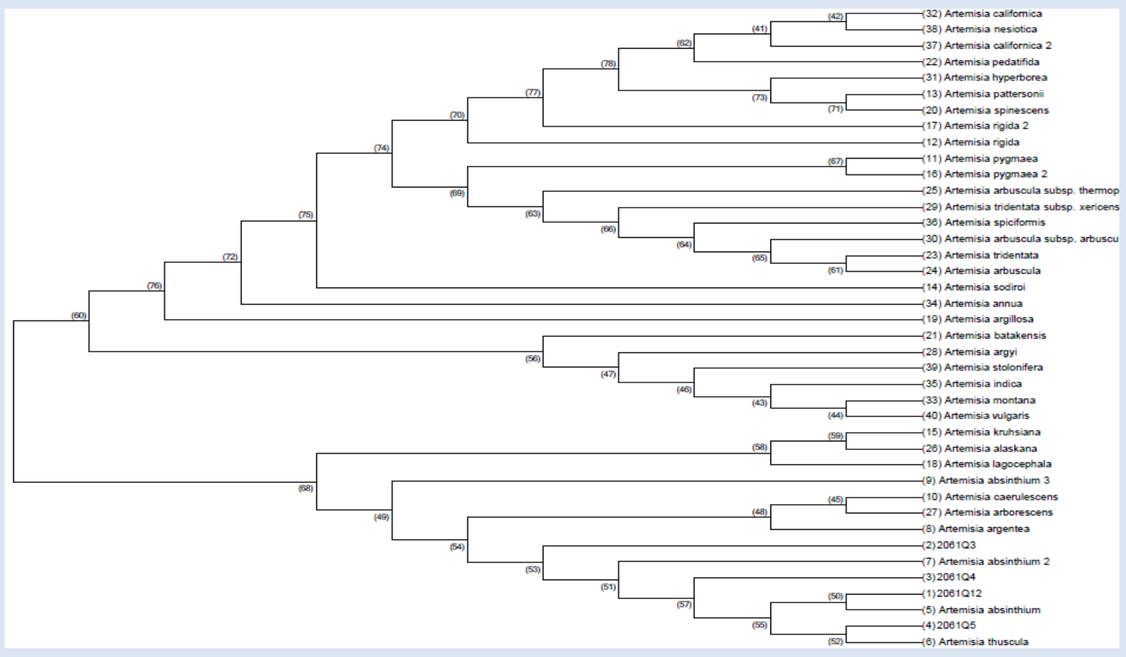

Figure 2. Phylogenetic tree constructed by neighbour-joining method using ITS sequences. 2061Q3, 2061Q4, 2016Q5 and 2061Q12 represent four samples (out of ten) certainly associated to the species *Artemisia absinthium*.

Discussion and Conclusions

The results showed that:

- Chemical and Genetic analysis data were convergent about the major nature of the questioned vegetable matter;
- Gathered data from genetic markers applied strongly suggested association of four samples (out of ten) to the species *Artemisia absinthium*.

In fact, ITS2 is an ideal barcode to infer the phylogenetic relationships in *Artemisia* spp.[14], corroborating this last mentioned inference about overall nature of the material analyzed: *Artemisia absinthium.*

Notwithstanding high levels of genetic sequences similarities compared to certain species from genera *Sambucus* spp., *Clematis* spp., *Elymus* spp. and *Cynodon* spp., but given the low levels of representativeness of these plant groups in the databases and, principally, unavailableness of intensive phylogenetic studies performed previously for them, we could not reach secure conclusions about the identity of these samples.

Our data, however, are sufficient to ascertain that it is not the case of proscribed drugs, neither protected plants, in Brazil: in this case, *Artemisia absinthium*, *Sambucus* spp., *Elymus* spp., *Clematis* spp. and *Cynodon* spp. are probably associated to folk medicinal practices.

At this degree of complexity, it is the first report about an effective effort using an association between Forensic Botany and Forensic Chemistry techniques in order to assess the real nature of a suspected plant material in Brazil. Reiterated questions from prosecution led us to lay hand on DNA barcoding technique, which succeeded to clarify the major nature of the questioned vegetable matter sent to the laboratory. The combination of DNA barcoding with Forensic Chemistry, therefore, can offer an efficient solution to evaluate plant material adequately from now on.

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