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TECHNICAL NOTE

Application of direct PCR in a forensic case of yew poisoning

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Abstract Intoxications with yew (*Taxus* spp.) pose a challenge to forensic toxicology because a variety of *Taxus* ingredients have been associated with its toxic effects. To provide preliminary evidence in cases where plant material is available, we introduce a novel direct PCR assay for the detection of DNA traces from *Taxus* spp. This assay has been successfully applied to a forensic case of suicidal poisoning via ingestion of *Taxus* leaves. PCR primers were designed to target a sequence located in the internal transcribed spacer 1 (ITS1) of nuclear ribosomal DNA, which is well conserved among species of the genus *Taxus* and can, therefore, be exploited to discriminate between *Taxus* and other conifers. Because ITS1 exists as a multicopy sequence within the plant genome, the assay provides enough sensitivity to work with trace amounts that are below the DNA content of a single cell. Specificity of the assay was tested with DNA extracts from Taxaceae and selected representatives from other related plant families (Cephalotaxaceae, Cupressaceae and Pinaceae). When combined with the commercial Phire® Plant Direct PCR Kit (Finnzymes), the primers allowed application of a two-step cycling protocol (without the annealing step), and because direct PCR requires only little sample pre-treatment, results from PCR could be obtained within 1.5 h after analysis had begun. Direct PCR was performed with diluted gastric content from the forensic case. Amplification products of the expected size were purified and sequenced. Sequence data were subjected to Basic Local Alignment Search Tool analysis and affiliated with ITS1 from *Taxus* spp.

Keywords Direct PCR · *Taxus* · Yew · Internal transcribed spacer 1 (ITS1) · Toxic plant poisoning

Introduction

Yews (*Taxus* spp.) are evergreen conifers that have been implicated with animal [1, 2] and human poisonings [3, 4]. Some *Taxus* species and cultivars are commonly used in ornamental horticulture and landscaping. All species and all parts of these plants are considered poisonous, except the sweet-tasting red aril [5], and because of their well-reputed toxicity, availability and simplicity of application, yews constitute a classical way of committing suicide to date [3].

Ingestion of *Taxus*-derived materials (leaves, seeds, etc.) can cause dizziness, nausea, vomiting, diffuse abdominal pain, cardiac arrest, respiratory paralysis and death [5]. A variety of *Taxus* ingredients have been associated with its toxic effects, including taxines (i.e. cardiotoxic alkaloids such as taxine B), taxanes (diterpens, e.g. the mitotic spindle poisons paclitaxel and docetaxel), nitriles (cyanogenic glycoside esters), ephedrine and (irritant) oils [5–8].

Several methods for the analysis of *Taxus* ingredients have been published, including immunoassay, thin layer chromatography, gas chromatography (GC), GC combined with mass spectrometry (MS), high-performance liquid

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chromatography combined with UV, diode array detection and MS [4]. But the toxicologically relevant content and composition of *Taxus* spp.-derived materials vary widely depending on the species (respective cultivar) [9, 10], the habitat (climate), the season and the type of sample [11–13]. In addition, very little is known about the toxicokinetics (liberation, absorption, distribution, metabolism and excretion), toxicodynamics and post-mortem fate of these components [14]. Thus, toxicological confirmation of *Taxus* spp. poisoning may prove to be difficult.

In order to avoid these complications and to provide preliminary evidence in cases of alleged yew intoxication, we introduce a novel DNA-based assay for the rapid and specific detection of cellular traces from members of the genus *Taxus* by PCR. The PCR assay amplifies a fragment of the internal transcribed spacer 1 (ITS1) which is part of the nuclear ribosomal DNA (rDNA) cistron from *Taxus* spp. ITS is popular and widely used in plant taxonomy as a molecular marker with a high sequence variability that is thought to reflect evolutionary distance at least to some degree [15]. However, the target sequence of the novel PCR assay is relatively well conserved among members of the genus *Taxus* and can, therefore, be utilized for the discrimination between *Taxus* and other conifers.

Materials and methods

Reference plant materials

Young, healthy branches were collected from classified plants (Taxaceae, Cephalotaxaceae, Cupressaceae and Pinaceae) at the Botanical Garden of Vienna and stored frozen at −20°C until DNA extraction. For further details, refer to the Electronic supplementary materials (ESM 1).

Primer design

Primers were designed to detect the ITS1 region of rDNA from *Taxus* spp. using Primer-Basic Local Alignment Search Tool (Primer-BLAST) [16] and sequence data from *Taxus baccata* (National Center for Biotechnology Information (NCBI) GenBank EF680246.1). Published rDNA sequences from *Taxus* species were aligned with Clustal X software (version 2.0, [17]) to confirm the conservation of primer binding sites among members of the genus (for details, see ESM 1).

DNA extraction

DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the supplier's instructions (for details, see ESM 1).

Sample preparation for direct PCR

Sample preparation for direct PCR (dilution protocol) was performed according to recommendations of the manufacturer of the Phire® Plant PCR Kit (Finnzymes, Woburn, MA, USA). Samples (cut leaves or aril) were placed in 20 µl of dilution buffer (provided with the Phire® Plant PCR Kit), crushed with a pipette tip, subjected to vortexing (1 min at maximum speed) and a subsequent centrifugation step (14,000×*g*, 1 min). Two microlitres of the supernatant was used as a template for a 20-µl PCR reaction.

Alternatively, a direct protocol (see ESM 1) may be applied. Both protocols have been successfully tested for direct PCR with sample materials from *T. baccata* (data not shown). However, for this study, only the dilution protocol was applied because it appeared to be more efficient in terms of amplicon yield and required no intermittent cleaning of the puncher.

PCR

PCR was performed in a total volume of 20 µl consisting of 1× Phire® Plant PCR buffer, template DNA, 0.5 µM of each primer (MWG Biotech, Germany; Table 1) and 0.4 µl Phire® Hot Start II DNA polymerase (Finnzymes). As recommended by the supplier of the direct PCR kit, melting temperature (Tm) values of primers were calculated with the Tm calculator on Finnzymes' website (http://www.finnzymes.com) based on the method by Breslauer et al. [18]. Calculated Tm values for both primers were higher than 72°C (Table 1). Therefore, a two-step PCR protocol could be applied where primer annealing and extension both occur in a single step at 72°C. PCR amplification was carried out on a 9700 GeneAmp Thermal Cycler (Applied Biosystems, Foster City, CA, USA) and included an initial denaturation step at 98°C for 5 min, followed by 40 cycles for 5 s at 98°C, 20 s at 72°C and finished with an extension step for 1 min at 72°C. Five-microlitre aliquots from PCR reactions were separated by agarose gel (1%) electrophoresis in 1× Tris–borate–EDTA buffer. Bands were visualized with UV light. Control primers (supplied with the Phire® Plant PCR Kit, Finnzymes; [19]) were used to confirm the presence of amplifiable DNA (for details, see ESM 1).

DNA sequencing

PCR products were inspected by agarose gel electrophoresis, purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced using BigDye Terminator sequencing reagents (version 3.1; Applied Biosystems), following the recommendations of the manufacturer (for details, see ESM 1).

Table 1 *Taxus* spp. primer sequences, binding positions on DNA, NCBI accession number of target reference sequence and melting temperatures

Primer	Orientation	Position (EF680246.1)	Tm (°C)[a]	Sequences (5′–3′)
*Taxus*_F	Forward	nt 492–511	75.85	TTTGGACGGGTGCACCTGCG
*Taxus*_R	Reverse	nt 677–696	76.32	TGCAATCCGCACCGATGCGT

Tm melting temperature

[a] Tm values calculated with the Finnzymes' Tm calculator according to [18]. The cycling protocol for PCR with the Phire® Hot Start II DNA polymerase was based on these calculations

Analysis of case samples by direct PCR

A small aliquot (~0.15 g) of sample material (gastric content from the forensic case; the case history can be found in ESM 1) was crushed with a sterile blade and placed into a 50-µl DNA Release® Additive (Finnzymes). The sample was vigorously vortexed for 1 min, briefly spinned down and then diluted with 0.5 ml water (Promega). After another minute of vortexing, the sample was subjected to centrifugation (14,000×*g*, 1 min). An aliquot from the resulting supernatant was used to prepare diluted samples (10-fold, 100-fold). Five-microlitre aliquots from the supernatant and the diluted samples were used as templates for PCR.

Results

Sensitivity

PCR was performed using diluted DNA extracts from *T. baccata* as templates in amounts ranging from 10 ng to 0.1 pg. Five-microlitre aliquots from PCR reactions were analysed by agarose gel electrophoresis (see Fig. 1 of ESM 2). Amplicons obtained with 1 ng template DNA were purified and sequenced. Sequence data were subjected to BLAST analysis and affiliated with ITS1 from *T. baccata* (data not shown). Notably, all tested amounts of template were sufficient to produce amplicons of the expected size (205 bp). Amplification with 0.1 pg template DNA produced a faint but visible band on agarose gel with a signal intensity comparable to that of about 20 ng from the 200-bp DNA marker band (MassRuler, MBI Fermentas).

Specificity

The specificity of the PCR primers was tested in vitro with diluted DNA extracts from species of the genus *Taxus* (*T. baccata*, *T. cuspidata*) and related conifers (Taxaceae: *Torreya nucifera*; Cupressaceae: *Cephalotaxus harringtonia*, *Taxodium distichum*, *Sequoia sempervirens*, *Cryptomeria japonica*, *Thuja plicata*; Pinaceae: *Abies fraseri*) at template concentrations of 1 ng (see Fig. 2 of ESM 2). In addition, extracts from the following conifers were tested applying the same experimental conditions: Taxaceae: *T. baccata* (samples harvested from four different individual plants), the cultivars *T. baccata* "fastigiata", *Taxus media* "hicksi"; Cupressaceae: *Chamaecyparis lawsoniana*, *Sequoiadendron giganteum* and Pinaceae: *Tsuga canadensis* (data not shown). PCR products were consistently obtained with template DNA originating from members of the genus *Taxus*, but no cross-reactions were observed with DNA from other plants. To ensure the presence of amplifiable DNA, control PCR reactions were performed with "universal" plant (chloroplast) primers that were supplied with the Finnzyme kit and all samples produced amplicons (data not shown).

Mixed sample (human–*T. baccata*) DNA analysis

Twenty-picogramme aliquots of diluted DNA extracts from *T. baccata* were used as templates for PCR—either pure or mixed with human DNA (20 pg to 200 ng). In addition, 200 ng of human DNA was subjected to PCR in a separate reaction. PCR was performed as described above. Amplicons were inspected by agarose gel electrophoresis (see Fig. 3 of ESM 2). Importantly, amplification of the *Taxus*-specific product appeared not to be grossly affected by the presence of human DNA, even when co-amplified with a 10,000-fold excess of human template. Two additional amplicons (estimated sizes of 0.4 and 0.5 kb, respectively) were reproducibly and specifically amplified only when human DNA was added. But these "human-specific" amplicons could be clearly distinguished from the *Taxus*-specific amplicons by size differences. Furthermore, the "human-specific" amplicons were clearly detectable only with very high amounts of human template DNA (20 ng or higher), which implies that the sensitivity for the human targets is very low compared to that for *Taxus* ITS1.

Analysis of case samples by direct PCR

Direct PCR was performed with diluted gastric content from the forensic case. PCR products of the expected size were generated with aliquots of diluted gastric content and inspected on agarose gel (see Fig. 4 of ESM 2). PCR

products were purified and sequenced. Sequence data were subjected to BLAST analysis and affiliated with ITS1 from *Taxus* spp. (a reference sequence can be found in ESM 3).

Discussion

Forensic toxicological analysis of intoxications with yew is challenging because a mixture of unstable alkaloids—in particular taxine B and taxine B-related compounds—seem to play a major role in the cardiotoxic effects associated with poisonings by yew plants [5]. The taxines can only be identified and quantified with highly sophisticated analytical techniques such as liquid chromatography–mass spectrometry [20]. Because appropriate reference substances and the necessary instruments are not available in most forensic laboratories, absorption of yew ingredients is often determined indirectly by high-performance liquid chromatography or gas chromatography–mass spectrometry via the marker 3,5-dimethoxyphenol (3,5-DMP, the aglycon of the *Taxus* ingredient taxicatine) [21]. However, this analytical approach has raised some criticism [22], mainly because 3,5-DMP is not considered responsible for the main toxicological effects and it is not yew-specific, as other plant sources (e.g. grapes) exist for this marker as well.

On the other hand, in most cases of accidental or intentional yew poisonings, yew fragments can be found on the scene and/or in the gastrointestinal tract. The novel approach of applying direct PCR to this material can also provide preliminary evidence in such case within a shorter time frame. Therefore, this approach could be used as an additional or alternative marker that can be established in forensic molecular biological facilities.

Taxus leaves are known to contain potent antifungal compounds (e.g. taxinines) [23] and, concluding from our own observations, they seem to be quite resistant to microbial decay. In spite of the fact that the case material (stomach content including crushed or partially crushed *T. baccata* leaves) used in this study had been stored at 4°C for about 1 year before PCR, the material was easily recognizable as coniferous “needles” in both sight and smell and, most importantly, it was unproblematic as a sample for direct PCR.

The analytical sensitivity of the PCR assay was tested with diluted DNA from *T. baccata*. As little as 0.1 pg template DNA was sufficient to generate detectable amounts of PCR products. Thus, when considering the reported *C*-value (i.e. the amount of DNA contained within a haploid nucleus) of 11.05 pg for *Taxus* [24], the PCR assay is sensitive enough to detect DNA traces in quantities that are clearly below the content of a single *Taxus* cell. This finding was expected because the targeted ITS sequence is part of the nuclear rDNA cistron, which is present in high copy numbers in most eukaryotes, including plants [15].

Currently, DNA barcoding receives much attention as a molecular approach to aid plant taxonomy, and it has been proposed as a powerful tool in forensic botanical investigations [25, 26]. Very recently, a study has been published that evaluates the utility of several genetic loci for species discrimination and identification of Eurasian yews [27]. Among others, the nuclear ribosomal DNA ITS1 region of *Taxus* was particularly recommended as a sequence with relatively high species discriminatory power. However, binding sites for the novel *Taxus* spp. primers used in the present study are well conserved among members of the genus and ITS1 was chosen as PCR target not least because of its discriminatory power at higher taxonomic (genus and above) levels [15]. With respect to the controversial taxonomic history of the genus *Taxus* [27], it may also be important to note that this novel PCR was a priori designed as a screening test to aid forensic investigations by detecting the presence of cellular traces from *Taxus* spp. and not to address complex phylogenetic tasks. Due to its relatively small amplicon size (205 bp)—a feature that is typically aspired in the field of molecular forensics (e.g. for the analysis of degraded DNA)—it simply does not have the discriminatory power of the entire ITS region (1,120 to 1,158 bp for Eurasian yews) or even ITS1 (733–745 bp) alone.

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