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The development of a DNA analysis system for pollen

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Abstract. The DNA analysis technique tRFLP (terminal restriction fragment length polymorphism) was utilised to develop an alternative to the traditional microscopic examination of pollen in order to facilitate the use of this valuable forensic evidence type. The gene chosen for this study was the alcohol dehyrogenase 1 gene. Statistical analysis established that the tRFLP technique was a reliable and reproducible technique that could provide considerable discriminating power between both plant and pollen species. This indicates that the tRFLP could be a suitable technique for the analysis of pollen communities as forensic evidence. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Forensic palynology, the use of pollen grains and spores as forensic evidence, was established in 1959 and has been used successfully in many cases [1]. The traditional method of pollen analysis involves high power microscopy where pollen grains are identified by the distinct patterns on the pollen wall [2]. The aim of our work was to develop a DNA analysis system for pollen that was sufficiently rigorous for forensic application.

We have utilised the tRFLP method to do this [3,4], using the alcohol dehydrogenase 1 (A*dh1*) gene. The ADH gene family is well studied and the A*dh1* gene is highly conserved [5] and present in all plants and pollen.

2. Materials and methods

DNA was extracted from control plant material (trumpet daffodil, *Narcissus* sp. tazetta daffodil, *Narcissus* sp., gerbera, *Gerbera jamesonii*, calla lily, *Zantedeschia aethiopiea*,

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kowhai, *Sophora microphylla*, poroporo, *Solanum laciniatu*, kakabeak *Clianthus puniceus* and Houpara, *Pseudopanax lessonii*), using the DNeasy® Plant Mini kit from Qiagen.

DNA was extracted from 50 mg pollen from trumpet daffodil, tazetta daffodil, gerbera, and calla lily using an organic extraction method containing 1 mm glass beads, a method designed to prevent shearing of the pollen DNA as the pollen coat was breached [6]. RNA was removed by the addition of 25 ug/ml DNA free RNase A and incubation at 37 °C for 30 min.

A*dh1* primers were those previously reported [7]. The primers were labelled with FAM, and were supplied by Invitrogen™ Life Technologies.

PCR amplification using Amplitaq Gold® PCR mastermix (PE Applied Biosystems), 300 nM of forward and reverse primers and 10 ng of DNA was under the following conditions: 95 °C, 10 min, then 94 °C, 1 min; 42 °C, 2 min; 72 °C, 4 min, for 40 cycles followed by a 30 min extension time at 72 °C.

Amplified products were precipitated with ethanol and digested with *Msp1* (New England Biolabs Inc). The restriction products were separated by size on the ABI Prism® 3100 genetic analyser using GS500 Rox size standards from PE Applied Biosystems). Fragment analysis was with Genescan® analysis version 3.7.

3. Statistical analysis

The base-pair size and peak area was used to assess the tRFLP peaks on the resultant electropherograms in order to determine the degree of difference (discrimination) between plants and pollen of different species.

Euclidean distance measures were used to describe the variation between any two samples being compared. A Euclidean value of 0.03 was determined from the data as a value at which no false inclusions would occur. Above this value, samples were considered to be different and below this value the samples would be considered to be the same.

Principle component analysis (PCA) was used to demonstrate the differentiation between species.

Linear discriminant analysis (LDA) was used to determine how likely it would be that an unknown sample would be correctly classified when compared to the data.

4. Results

Young leaves were sampled from the eight plant species and DNA extracted from five replicates. Of the 40 samples extracted, 4 samples gave no profiles at all and 1 (from a kowhai leaf) was different on visual inspection to the other profiles obtained from the same plant species. Pair wise comparison of the samples from all of the species tested using a Euclidean distance measure showed that the tRFLP technique had considerable discriminating power and could be used successfully to determine whether two samples may have come from the same origin. For example, within species comparisons gave very low values. From 61 within species comparisons, a distance measure of below 0.01 was calculated 93% of the time. Conversely, between species comparisons gave high values. From 573 between species comparisons a distance measure of above 0.04 was calculated 99.5% of the time.

PCA showed good separation of the different species and LDA demonstrated that classification of an unknown sample, as a particular species, was 97% successful.

Intra-specific species variation was investigated by comparing five replicates of each of three different calla lily plants. All replicates gave very small Euclidean values bar one. This one replicate, with a value of 0.069 fell outside the range and would have been falsely excluded by the selected criteria.

Having established that the tRFLP method could distinguish between different plant species, it was then tested on pollen isolated from four of the control plants, two daffodil species, gerbera and calla lily. Duplicate analyses of each pollen sample showed good reproducibility. Substantial variation was found between profiles from pollen from different species. Euclidean values ranged from 0.06 to 0.20. Calla lily pollen from three different plants was used to assess intra-specific variation. Twelve out of 15 comparisons generated Euclidean values of less than 0.03, indicating that these samples could be correctly included as having a common species source. Visual inspection of the remaining DNA profiles revealed the presence of several small peaks absent from the others. This lack of consistency is cause for concern but leads to false exclusion.

When the pollen profiles were compared to the plant profiles from the same plants, those of the two *Narcissuss* sp. were statistically similar whereas significant differences were observed for the other species indicating that plant material should not be used as a reference sample for a particular pollen sample.

5. Discussion

These analyses showed that the tRFLP DNA analysis technique was successful in providing good discrimination between species and high reproducibility within species. When comparing different plant species, no false inclusions were observed. The false exclusion that was observed may be a result of degraded DNA and this needs further investigation. Similar results were obtained from the study of pollen samples. Overall these results indicate that tRFLP is a suitable technique for the analysis of pollen communities as forensic evidence.

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