Analytical Methods

Development of primer and probe sets for the detection of plant species in honey

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ABSTRACT

An objective of workpackage 3 within the European Research Project "TRACE" was the identification of PCR markers for the detection of plant species related to honey. Within the project "Miel de Corse", a protected designation of origin (PDO) honey and "Miel de Galicia", a protected geographical indication (PGI) honey as well as German and English honeys were analysed. Beside the development of plant species specific real-time PCR systems a method has been established for the detection of DNA derived from plant species in general.

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1. Introduction

European Regulation (EC) No. 178/2002 entered into force on 1st January 2005, and included important elements on the rules for traceability and the withdrawal of potentially dangerous food products from the market. To enable the food control authorities to supervise compliance with these labelling requirements, suitable detection methods must be available. The study presented here was prepared within workpackage 3 of the European Research Project TRACE. This workpackage aimed to develop rapid, robust, accurate and cost-effective methods for determining the species/varietal origin of food using DNA based methodology. This methodology is uniquely suited for this application due wholly to the specificity and sensitivity afforded by the polymerase chain reaction (PCR), which allows the unambiguous identification of animal and plant species in food. The unique specificity and selectivity of PCR is extended by the utilisation of real-time PCR, where the exponential amplification of target specific DNA is measured using dual labelled fluorescence probes (TaqMan™ technology) (Holland, Abramson, Watson, & Gelfand, 1991) and a signal is only observed if the target species is present. This technique offers significant advantages for routine analysis since the assay is rapid and a large number of food samples can be analysed in a single run.

The objective of the present study was to develop methods which could authenticate 'Miel de Corse', a product of protected designation of origin (PDO). Products of PDO and products of protected geographical indication (PGI) were adopted by the European Commission (EC) in Regulation (EEC) No. 2081/92, in 1992. Subsequently, there was an application for the registration of "Miel de Corse" as a PDO, pursuant to Article 6 (2) 1999/C 239/02 of Regulation 2081/92, where honey eligible to use the registered designation of origin 'Miel de Corse' may only be produced from a geographical area encompassing the island of Corsica.

To date, the origin of a honey has been confirmed by the microscopical analysis of pollen (Louveaux, Maurizio, & Vorwohl, 1978). The spectrum of pollen varieties indicates the plants visited by bees during the production of the honey and permits the characterisation of the geographic origin. However, this type of analysis is dependent on the experience and skill of highly trained analysts. In contrast, DNA based analytical methods are less dependent on the analyst and can be applied in different laboratories equipped with suitable instruments. Additionally, DNA based methods are suitable for standardisation as a basis for harmonisation throughout the European Union and potentially beyond.

The study focused on the development and validation of real-time PCR systems for the detection of plant species commonly found on Corsica: sweet chestnut, lavender, eucalyptus, rockrose, oak and broom, which should be used to build a profile of the

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plants used by bees as forage during the production of "Miel de Corse". Additionally, detection systems using real-time PCR for other plant species commonly identified in honey (acacia, linden, citrus, clover, heather, olive, rape, sunflower and rosemary) were also developed and validated. These real-time PCR systems were then used to distinguish Corsican honey from samples of honey from other geographic regions.

2. Materials and methods

DNA from acacia (*Acacia decora*, *Acacia longifolia*), almond (*Prunus dulcis*), blackberry (*Rubus fruticosus*), broom (*Cytisus scoparius*), chestnut (*Aesculus hippocastanum*), citrus (*Citrus grandis*, *Citrus limon*, *Citrus sinensis*), clover (*Trifolium alpestre*, *Trifolium medium*, *Trifolium montanum*, *Trifolium rubens*), heather (*Erica carnea*), eucalyptus (*Eucalyptus erythrocorys*), hazelnut (*Corylus avellana*), lavender (*Lavandula angustifolia*, *Lavandula lanata*, *Lavandula latifolia*, *Lavandula stoechas*), linden (*Tilia platyphyllos*), maize (*Zea mays*), melissa (*Melissa officinalis*), oak (*Quercus palustris*, *Quercus pubescens*, *Quercus robur*, *Quercus rubra*, *Quercus suber*), olive (*Olea europaea*), papaya (*Carica papaya*), rape (*Brassica napus*), rockrose (*Cistus ladanifer*, *Cistus monspeliensis*), rosemary (*Rosmarinus officinalis*), sage (*Salvia fruticosa*, *Salvia lavandulifolia*), sunflower (*Helianthus annuus*), sweet chestnut (*Castanea sativa*), thyme (*Thymus vulgaris*) and wheat (*Triticum aestivum*) was extracted from plant material (leaves, flowers or pulp) purchased from the Botanical Garden in Berlin, Germany or from commercially available plants. The plant material was ground in liquid nitrogen using the A11 Basic Analytical Mill (IKA, Germany) and stored at −80 °C. The DNA was extracted using a cetyltrimethylammonium bromide (CTAB) method based on the procedure published by Tinker, Fortin, and Mather (1993). DNA extracted from cattle (*Bos taurus*) and chicken (*Gallus gallus*) were used as negative controls.

Twelve to 15 different honeys were collected from each of four regions; Corsica, Spain (Galicia), Germany and the UK (Table 1). Only four regions were considered due to the wide variety found within each. These products were obtained in the appropriate countries from commercial sources or directly from the beekeeper. It was assumed that these honeys had not be adulterated or extended with material from other sources.

The extraction of DNA from honey described below was optimised and validated in a small-scale comparison with four independent laboratories. Honey (10 g) was diluted with water to 45 ml, incubated at 65 °C in a water bath and shaken for 30 min. Following centrifugation for 30 min at 2400g, the pellet was resus-pended in 200 μl water and ground using a 3 mm Tungsten Carbide bead in a TissueLyser (QIAGEN GmbH) for 2 min at 30 Hz. Using the DNeasy Blood and Tissue Kit (QIAGEN GmbH) the sample was digested for 30 min at 56 °C in 50 μl of buffer Al with 25 μl of proteinase K. The remainder of the DNA extraction was carried out exactly according to the manufacturer's instructions (DNeasy Blood and Tissue Kit Handbook July 2006). The DNA concentrations of samples were determined by spectrophotometry (DynaQuant 200, Hoefer, Amersham Bioscience).

Candidate genes for TaqMan™ PCR were identified and sequence data obtained from the GenBank database (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2005) (Section 3) or where no sequence entry was available (*actin*, *tRNA-Leu* (trnL), *HMG*2 and *adh*1) by DNA sequence analysis using consensus primers. With the exception of the primer pair used to amplify the *tRNA-Leu* (trnL) gene fragment (Taberlet, Gielly, Pautou, & Bouvet, 1991), all of the primers used were developed within this project (Table 2).

Conventional PCRs were performed in a total volume of 25 μl. The reaction mixture contained 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.2 μM of each primer (listed in Table 2), 1 × PCR buffer (GeneAmp® PCR buffer, PE Applied Biosystems), 2 mM MgCl$_2$, 1.5 units Taq polymerase (AmpliTaq Gold™ DNA Polymerase, PE Applied Biosystems), and 2 μl (10–50 ng) of template DNA solution. Amplification was performed in the Thermal Cycler GeneAmp PCR System 9700 (Applied Biosystems) with the following thermal cycling protocol: 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, an annealing step for 30 s, 72 °C for 30 s and a final single step of 72 °C for 6 min. The annealing temperature used for the respective systems was determined empirically to be 60 °C, with the exception of sweet chestnut (58 °C), sunflower (56 °C), rape (57 °C), linden (58 °C), clover (58 °C) and the general actin-system for plants (57 °C).

TaqMan™ PCRs were performed in a total volume of 10 μl. The reaction mixture contained 1 × TaqMan™ Buffer A, 1.5 units AmpliTaq Gold® Polymerase, and magnesium chloride buffer (all from the Applied Biosystems TaqMan™ PCR Core Reagent Kit), 0.2 mM of each dNTP, forward and reverse primers, dual labelled fluorescent probe, and 2 μl (5–25 ng) of template DNA solution. The final concentration of the primers, probe and magnesium chloride in the respective systems was optimised empirically (Table 3). The primers and probes were purchased from TIB MOLBIOL (Germany) or Sigma (UK) and are listed in Table 2. The high magnesium chloride values determined after optimisation, might be due to the comparatively low melting temperatures of some primers and the need for probe stabilisation in TaqMan™ assays (Applied Biosystems 7700

Table 1
Honey samples ordered by country of origin

Corsica	Galicia	Germany	UK
Automne	Brezo	Blütenhonig (multi-floral)	English
Chataigneraie	Brezo	Edelkastanie (sweet chestnut)	English blossom
Maquis de Printemps	Castano	Edelkastanienhonig (sweet chestnut)	English set multi-floral
Maquis du desert des agriates	Eucalypto	Frühjahrsblüte (multi-floral, spring)	English set multi-floral
Miel de Bruyere (heather)	Galicia	Lindenhonig (linden)	English set multi-floral
Miel de Corse	Multi-floral	Lindenhonig (linden)	Exmoor heather
Miel de Corsica	Multi-floral (mainly chestnut and oak)	Raps (rape)	Heather
Miel de Printemps	Multi-floral (mixed heather + eucalyptus)	Rapshonig (rape)	Multi-floral
Miel du Maquis	Multi-flower (mainly heather)	Robinie (robinia)	North-Yorkshire multi-floral
Miel du Maquis d ete	Single-floral chestnut	Sommerblüten mit Kornblume (multi-floral, sommer)	Scottish heather
Miellats du Maquis	Single-floral eucalyptus	Sonnenblumenhonig (sunflower)	Yorkshire chunk multi-floral
Miellats du Maquis	Single-floral eucalyptus	Vielblütenhonig (multi-floral)	Yorkshire spring-blossom multi-floral
Printemps "Asphodele"	Single-floral eucalyptus	Waldhonig (forest)	
Printemps clementinier	Single-floral heather	Weißtanne (white fir)	
Printemps mille fleurs	Single-floral heather		
Printemps oranger	Single-floral (partly blackberry)		

Table 2
Specifications of primer and probe systems

Species	Gene and accession number if available	Oligonucleotide	Name	Sequence 5′–3′	Amplicon length (bp)
Acacia	*Alcohol dehydrogenase* 1 (*adh*1)	Forward primer Reverse primer Probe	Aca_adh1-f Aca_adh1-r Aca_adh1-probe	TGG GGA ATG TCC ACA TTG TAA GT TGG AGA ACC TGG TCT TGC CA FAM-AGC ATA ACA CCC CTG TCA GTG TTA ATC CTA AGA A -TAMRA	107
Broom	*Actin*	Forward primer Reverse primer Probe	Cyt_act-f Cyt_act-r Cyt_act-probe	GTT CAC CAC CTC TGC TGA GCG A TCG GCT CCA ATT GTG ATA ACT TGT FAM-CAA GAG CAG TTC TTC AGT TGA GAA AAG CTA TGA -TAMRA	160
Citrus	*Alcohol dehydrogenase* 1 (*adh*1)	Forward primer Reverse primer Probe	Cit_adh1-f Cit_adh1-r Cit_adh1-probe	TGA CAG AGG CGT CAT GCT TAA C TGA GGT TCC AAC GAA ATG ATA AAT A FAM-CTT GCC ATT GAT GGA AAA TCT CGA TTT CC -TAMRA	83
Clover	*Actin*AY372368 (*Trifolium pratense*)	Forward primer Reverse primer Probe	Tri_act-f Tri_act-r Tri_act-probe	CTG GCC GTG ATC TAA CTG AAT CTT ATC AGG AAG CTC ATA GTT TTT CTC AAT T FAM-AAG TTC TTG TTC ATA ATC CAC AGC AAC ATA GGC AA -TAMRA	191
Heather	*Alcohol dehydrogenase* 1 (*adh*1)	Forward primer Reverse primer Probe	Eri_adh1-f Eri_adh1-r Eri_adh1-probe	GGA TAG GGG AGT GAT GAT CCA T GAT GTT CCG ACG AAA TGG TAT ATG FAM-TGC CGT TTT TAG AAA ACC TTG ATT TCC CA -TAMRA	81
Eucalyptus	*Leafy/Floricauly* (*LFY*1)AY640313 (*Eucalyptus grandis*)	Forward primer Reverse primer Probe	Euc_LFY-f Euc_LFY-r Euc_LFY-probe	AGG AGC AAG TGG TGC AGC A TCC TCT GTT GTT GGG CGC FAM-TCA GAA AAG GAT CAG CTG GGC AGG G -TAMRA	96
Lavender	*Hydroxymethylglutaryl coenzyme A reductase* (*hmg*)	Forward primer Reverse primer Probe	Lav_HMG2-f Lav_HMG2-r Lav_HMG2-f-probe	CCA TAT CGC TCG TCT TCA ACA G TTG CCG GCG ATG GCA FAM-CAC TTG ATG CTC TGA AGC TTG GCG A -TAMRA	74
Linden	*Nitrate reductase* (*nr1*)AY138811 (*Tilia platyphyllos*)	Forward primer Reverse primer Probe	Til_nr-f Til_nr-r Til_nr-probe	GGG CAT CGA ACA TGA GCT TT TTC AAC GAG TTT GCA TGG GA FAM-AAA GAA GAT GCG CCT ACA AGA CCT GTT GC -TAMRA	101
Oak	*Phenylalanine ammonia-lyase* (*PAL*)AY443341 (*Quercus suber*)	Forward primer Reverse primer Probe Forward primer	Querc_PAL-f Querc_PAL-r Querc_PAL-probe Querc_PAL2-f	AGT AGA GGG CGC AAG GAT AGA AA GAA TTC TCT CAC CAG TTA GTA AAC TTG TT FAM-AAA GCA GCA ATT CCT AAC CCA ATT AAG GAG T -TAMRA AAG AAG TAG AGG GCG CAA GG	136 141
Olive	*Ole e 10*AY082335 (*Olea europaea*)	Forward primer Reverse primer Probe	Olea_ole10-f Olea_ole10-r2 Olea_ole10-probe	TAC CAA AGC AAG GGT CGA AAT G AGG GAT AAA ATT GTT TTA CTT ACT TGG GT FAM-TCG ATT GCG ACT TTT CGG GCA CC -TAMRA	96
Rape	*Lipase 1*AY866419 (*Brassica napus*)	Forward primer Reverse primer Probe	Bras_lip-f2 Bras_lip-r Bras_lip-probe	CAC GAC CAC GTT CTT TGT TTT CCA CTA CAA CAT TCC ATC CCC FAM-TCA CCA AAA CCG CAG CAA GCA -TAMRA	122
Rockrose	*1-Deoxy-D-xylulose 5-phosphate reductoisomerase* (*DXR*)AY297794 (*Cistus incanus*)	Forward primer Reverse primer Probe	Cis_DXR-f Cis_DXR-r Cis_DXR-probe	AAT GTT CGA TGT CCT CCT GTT CTT GCT CAC TGT TTC CAC CCA CAA FAM-CCA ATG GAT CTC TCA ATG GCG CTG TC -TAMRA	94
Rosemary	*Alcohol dehydrogenase* 1 (*adh*1)	Forward primer Reverse primer Probe	Ros_CSL-f Ros_CSL-r Ros_CSL-probe	GCG TAT GCG GCC AAC AG ACT GCC CTT GAA GAA GAA ATG G FAM-TGG CAG ATT CAT CAT TCT CCT TCA TTA GGA CC -TAMRA	74
Sunflower	*Profilin*J15210 (*Helianthus annuus*)	Forward primer Reverse primer Probe	Heli-all-nes f2 Heli-all r1 Heli-all-nes probe	CGT CAA TAC TTG TTA ATA TTA TTA AGA ATT A ATA GCT TGG CCC GTT TTC TT FAM-ATG CAT ATT CCT CCA GCT CCC TG -TAMRA	106
Sweet chestnut	*ypr10 gene for allergen Cas s 1*AJ417550 (*Castanea sativa*)	Forward primer Reverse primer Probe	Cas-all-nes f Cas-all-nes r Cas-all-nes-probe	GAG TGC TGA AAT CAT TGA AGG AAA T AAT GAT GTG TTG GAG ATG AGA ATA GAA G FAM-AGG CCC CGG AAC CAT CAA GAA GAT C -TAMRA	101
	Phenylalanine ammonia-lyase (*PAL*)	Forward primer Reverse primer Probe	Cas_PAL-f Cas_PAL-r Cas_PAL-probe	CCA AAG AAG TAG AGG GTG CAA GA GAA TTC TCT CAC CAG TTA GTA AAC TTG TT FAM-AAA GCA GCA ATT CCT AAC CCA ATT AAG GAG T -TAMRA	144
Plant	*tRNA-Leu* (*trnL*) Taberlet et al. (1991)	Forward primer Reverse primer Probe	Plant nes-2-f Plant nes-2-r Plant nes-2-probe	ATT GAG CCT TGG TAT GGA AAC CT GGA TTT GGC TCA GGA TTG CC FAM-TTA ATT CCA GGG TTT CTC TGA ATT TGA AGT T -TAMRA	Approx. 90
	*Actin*AF111812 (*Brassica napus*), AF282624 (*Helianthus annuus*)	Forward primer Reverse primer Probe	Act-f Act-r Act-probe	CAA GCA GCA TGA AGA TCA AGG T CAC ATC TGT TGG AAA GTG CTG AG (FAM)-CCT CCA ATC CAG ACA CTG TAC TTY CTC TC -(TAMRA)	Approx. 103

Table 2 (continued)

Species	Gene and accession number if available	Oligonucleotide	Name	Sequence 5′–3′	Amplicon length (bp)
Sequencing primers	*trnL*	Forward primer	Plant 1-f	CGA AAT CGG TAG ACG CTA CG	
		Reverse primer	Plant 1-r	GGG GAT AGA GGG ACT TGA AC	
	adh1	Forward primer	Adh1-f	AGT GTT GGA GAG GGT GTG ACT GA	
		Reverse primer	Adh1-r2	CCA ACA TGC ATG ACR GTG TAC TC	
	hmg	Forward primer	HMG2-border-f-w	GGG CAG TGY TGT GAG ATG C	
		Reverse primer	HMG2-border-r-w	ATR CCG ATG ACA TCC ATG TC	
	Lipase 1	Forward primer	Bras_lip-f	CTG TTA CGC CTT TCC CAA GAA C	
		Reverse primer	Bras_lip-r	CCA CTA CAA CAT TCC ATC CCC	
	Profilin	Forward primer	Heli-all-nes f	GCG GGT GCA AAA TAT ATG GTA C	
		Reverse primer	Heli-all r1	ATA GCT TGG CCC GTT TTC TT	

R = A/G and Y = C/T (IUB code).

Table 3
Optimum concentration of primers, probes, and magnesium chloride (MgCl$_2$) for TaqMan™ PCR determined by titration

System	Forward primer (nM)	Reverse primer (nM)	Probe (nM)	MgCl$_2$ (mM)
Aca_adh1	300	300	200	8
Act	900	900	200	8
Bras_lip	900	50	200	6
Cas-all-nes	300	300	200	8
Cas_PAL	300	900	200	7
Cis_DXR	300	900	200	8
Cit_adh1	300	300	200	5
Cyt_act	500	500	200	5.5
Eri_adh1	300	300	200	8
Euc_LFY	300	900	200	5
Heli-all-nes	900	300	200	5
Lav_adh1	900	900	200	6
Lav_HMG2	300	300	200	6
Olea_ole10	300	900	200	4
Plant nes-2	300	300	200	7
Querc_PAL	300	300	200	3
Querc_PAL2	900	300	200	6
Ros_CSL	900	900	200	5.5
Til_nr	300	900	200	7
Tri_act	900	300	200	4

SDS Workshop). Amplification was performed on the Applied Biosystems 7900 HT Real-Time PCR FAST System, with the following thermal cycling protocol: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

3. Results and discussion

The aim of this study was to develop a set of assays which, when used together on honey samples from Corsica, would provide a profile of the plant varieties visited by foraging bees, during honey production. These profiles would be indicative of the habitat on Corsica and could then be used as a reference against which honey samples could be authenticated.

The set of assays was composed of two elements: (a) a generic plant assay as proof of the presence of plants and (b) species specific assays for the detection of plants commonly found on Corsica and the EU mainland.

3.1. Development of plant specific TaqMan™ PCR systems

To develop a generic plant PCR system, which would detect a diverse range of plant pollen varieties in honey, several candidate genes were examined. It was found that the *rubisco activase*, *hydroxymethylglutaryl coenzyme A reductase*, *profilin* and *alcohol dehydrogenase* genes did not show sufficient interspecies sequence similarity to be suitable for the design of a generic plant PCR system (data not shown). However, in contrast, the *tRNA-Leu* (trnL)

gene was found to be sufficiently similar between plant species and was, therefore used for the design of a generic plant PCR system (Table 2; plant nes-2), following in-house sequence analysis for a range of plant species, using primers previously shown to amplify a diverse range of plant species (Taberlet et al., 1991) (Table 2; plant 1-f, plant 1-r). This multicopy gene was particularly suitable for this application since there are very small amounts of pollen, and therefore DNA in honey and the use of a gene with more than one copy per cell increases the sensitivity of detection for TaqMan™ based assays. Fig. 1a shows the amplification plots of the generic primer and probe set using template DNA isolated from a variety of plant species.

Real-time PCR systems based on single copy gene sequences are central to the development of quantitative methods. To examine the possibility of applying a quantitative aspect to the characterisation of the plant content of honey, a PCR system (Table 2; act) based on a 103 bp region of the single copy gene *actin* was designed. When a range of plant species were tested using 5 ng DNA in a TaqMan™ PCR (Fig. 1b) the C_T values produced varied widely indicating that the assay could not be easily used to provide accurate quantitation. Factors contributing to the observed variation may include the presence of inhibitors, differing genome sizes, primer and probe sequence mismatches, and the degree of ploidy in differing species. To further complicate matters, it should be noted that the pollen profile of honeys can be season dependent, and that pollen from some plants can be strongly over or underrepresented in various honeys. For example, levels of lavender pollen in honey derived from lavender plant nectar are typically very low. These factors contribute to make the characterisation of honey by the determination of the exact amount of a plant species in relation to the total plant content using real-time PCR problematical. Hence the approach in this study to characterise the origin of honeys based upon the determination of qualitative plant species markers specific for geographic regions.

3.2. Species specific detection

The design of species specific primer and probe sets used a variety of strategies, dependant upon whether sequence information was available on the NCBI database or if *de novo* sequence analysis was required. Sequence information was available for clover, eucalyptus, linden, oak, olive, rape, rockrose, sunflower and sweet chestnut. The design of most of these assays was therefore, relatively straight forward (gene, accession number and primer and probe sequences given in Table 2). There was little or no DNA sequence information available for the design of acacia, broom, citrus, heather, lavender or rosemary specific primer and probe sets. *De novo* sequence was generated for each of these species which was then used to design species specific primer and probe sets. The *alcohol dehydrogenase 1* (*adh*1) gene was amplified using consensus primers (Table 2; adh1-f, adh1-r2) designed using DNA se-

Fig. 1. *Specificity test*: fluorescence curves for samples of acacia (–■–), citrus (–✕–), heather (–●–), rosemary (–◆–), lavender (–■–), sweet chestnut (–◆–), sunflower (–▲–), rape (–+–), linden (–□–), eucalyptus (——), clover (–▲–), broom (–●–), rockrose (–△–), oak (–✕–), and olive (–◇–) including cattle (–○–) and water (——) using the (a) plant nes-2, (b) act, (c) Aca_adh1, (d) Cit_adh1, (e) Eri_adh1, (f) Ros_CSL, (g) Lav_HMG2, (h) Cas-all-nes, (i) Heli-all-nes, (j) Bras_lip, (k) Til_nr, (l) Euc_LFY, (m) Tri_act, (n) Cyt_act, (o) Cis_DXR, (p) Querc_PAL and (q) olea_ole10 primer and probe system.

quence information from vine, sorghum, rice and barley. After DNA sequence analysis, specific primers and probes were successfully

designed for the detection of acacia, citrus, heather and rosemary (Table 2, Fig. 1c–f, respectively). However, the primers and probe

Fig. 1 (continued)

for the detection of *Lavandula stoechas* also amplified *Lavandula lanata* and *Lavandula latifolia*, although with significantly lower sensitivity (data not shown). An alternative PCR system was therefore developed, which would detect all lavender species with the same sensitivity. It was based on the *hydroxymethylglutaryl coenzyme A reductase* (*hmg*) gene for which sequence information for cotton, capsicum, tomato, potato and tobacco were available. After amplification and sequence analysis using consensus primers (Table 2; HMG2-border-f-w, HMG2-border-r-w) a specific primer and probe set was successfully designed which detected all lavender species with similar sensitivity (Table 2 and Fig. 1g). The detection system for sunflower was based on the *profilin* gene, however the NCBI database entry was for the mRNA and the amplicon size, for primers which had been designed to amplify a region 105 bp long, was found to be approximately 200 bp by gel electrophoresis. Sequence analysis of the amplicon (Table 2 for primers used:

Heli-all-nes-f, Heli-all r1) revealed an intron/exon structure and therefore a new primer was designed which would amplify a DNA region of 106 bp. Sequence information of primers and probe is given in Table 2 and Fig. 1h shows the amplification plot for a selection of samples. The detection system for rape is based on the *lipase 1* gene. The NCBI database entry was also for the mRNA and after sequence analysis (see Table 2 for primers used: Bras_lip-f, Bras_lip-r) primers were designed to amplify a DNA region 122 bp long.

3.3. Specificity of species specific TaqMan™ PCR systems

The specificity of these primer and probe systems in TaqMan™ PCR was assessed using template DNA isolated from 25 plant species and two animal species as listed in Section 2. There was no significant cross amplification detected for the primer and probe

systems specific for acacia, broom, citrus, clover, heather, eucalyptus, lavender, linden, olive, rape, rockrose, rosemary and sunflower. Exemplary amplification curves are shown in Fig. 1. However, it was found that the sweet chestnut and oak primer and probe systems (Cas-all-nes and Querc_PAL) each cross amplified the other's DNA. Sweet chestnut (*C. sativa*) and oak (*Quercus* sp.) are closely related: in the same subfamily *Quercoideae* and on analysis of the DNA sequence it was found that there was a high degree of sequence similarity between these plant species. Therefore to distinguish sweet chestnut from oak, two alternative forward primers were designed which recognised two base differences within the PAL fragment (Cas_PAL-f and Querc_PAL2-f, Table 2). These alterations resulted in a delta C_T value of approximately 13 between oak and sweet chestnut and about six between sweet chestnut and oak using the new alternative oak and sweet chestnut primers, respectively. These differentials equate to a reduced sensitivity of approximately 100- and 10,000-fold on the alternate template DNA, for the new oak and sweet chestnut assays, respectively, and indicate a non-relevant quantity of a plant species in honey since the total DNA yield from honey is very low as described in the next section. Fig. 2 shows the fluorescence curves for a selection of samples.

3.4. Sensitivity and efficiency of the TaqMan™ PCR systems

The limit of detection (LOD), efficiency and linearity of the systems were assessed to determine the suitability and capacity of each. Tenfold dilution series of template DNA were tested in 10 replicates for each system (data not shown). It was found that for all systems the LOD was at least 25 pg, where the probability of obtaining a C_T value lower than 45 was 95%. On average a C_T value of 37 was measured. The efficiency of the systems was calculated to be between 88% and 117 % according to (Vaerman, Saussoy, & Ingargiola, 2004)

$$E = 10^{-\frac{1}{s}} - 1$$

where *E* is the efficiency and *s* is the slope of the regression line for the log DNA concentration plotted against C_T value. This range of efficiencies was acceptable since although logically a reaction efficiency cannot be greater than 100%, the measurement uncertainty could result in a calculated efficiency greater than 100%.

The dynamic range of the systems, where there was a linear relationship between the template concentration and the C_T values returned, were shown to be in the order of at least 3 $\log_{10}$ dilutions, confirmed by the strong linear relationship between C_T values and the $\log_{10}$ of the starting quantity up to 2.5×10^5 pg ($r^2 > 0.99$) (data not shown). The repeatability for each system was determined by measuring duplicates of three dilutions of template DNA, on three different days. The results showed a relative standard deviation of up to 33 % (data not shown).

3.5. Application of the real-time PCR systems to honey

The real-time PCR systems were used for the detection of plant species in honey. DNA was extracted and purified from honey samples from four target countries: Corsica, Spain (Galicia), Germany and England. The countries were chosen to have different flora thereby providing unique plant profiles for the honeys from each country.

Initially the DNA extracts of all the honey samples were analysed using the generic plant system and it was found that plant DNA could be unambiguously identified in all honey samples investigated. However, plant DNA was only present in low amounts, and thus at or close to the LOD of the PCR systems. In detail, using the general actin-system for plants which detects a single copy sequence an average C_T value of 34 was measured, whereas the general plant system that detects a multicopy sequence produced an average C_T value of 29. Using the plant species specific primer and probe systems an average C_T value of 36 was measured.

Analysing the DNA extracts of all the honey samples using the species specific real-time PCR systems (Table 4) it was found that there were geographical differences in the range of plant species found in honeys from Corsica, Galicia, Germany and England. In particular it was found that:

- rape was not detected in Corsican and Galician honeys, but in almost all German and English honeys analysed to date;
- sweet chestnut was detected in almost all honeys from Corsica and Galicia, but only in a few German and English honeys;
- rockrose and olive were only detected in some Corsican honeys.

These results indicated that a combination of plant species specific real-time PCR systems could be used to determine the geographical origin of honey samples from Corsica, when compared to honey samples from Galicia, Germany and England. The systems which were most informative were sweet chestnut, rockrose, rape and olive. The chi-square test was used to analyse each of the 15 detection systems to determine whether frequencies of positive results differed with geographical origin. The Fisher's exact test was used where sample sizes were too small for the chi-squared test to be accurate (expected frequency less than 5). The results are shown in Table 4 where the significance is given in terms of the *p*-value: the bigger the difference the smaller the *p*-value and where a *p*-value less than 0.05 refers to a significant difference. The results listed in Table 4 confirm that there is no statistical

Fig. 2. Fluorescence curves for samples of acacia (–■–), citrus (–✕–), heather (–●–), rosemary (–◆–), lavender (–■–), sweet chestnut (–◆–), sunflower (–▲–), rape (–+–), linden (–□–), eucalyptus (–—–), clover (–▲–), broom (–●–), rockrose (–△–), oak (–✕–), and olive (–◇–) including cattle (–⊖–) and water (–—–) using the (a) Cas_PAL and (b) Querc_PAL2 primer and probe system.

Table 4
Statistical description for frequencies of positive results (%) using chi-square test or Fisher's exact test

Real-time PCR system specific for	Number of samples	Geographical origin of honey, percentage of positive results		p-Value
		Germany, UK, Galicia	Corsica	
Acacia	44	0	8	Not significant
Broom	58	5	0	Not significant
Citrus	37	0	30	0.015
Clover	47	29	0	0.019
Heather	58	17	44	0.043
Eucalyptus	58	2	0	Not significant
Lavender	53	0	13	Not significant
Linden	37	22	0	Not significant
Oak	58	7	25	Not significant
Olive	58	0	25	0.004
Rape	58	52	0	<0.001
Rockrose	58	0	63	<0.001
Rosemary	37	0	20	Not significant
Sunflower	53	5	0	Not significant
Sweet chestnut	58	57	60	Not significant

difference for Corsican honey versus German, English and Galician honeys for the frequency of the presence of DNA from acacia, broom, eucalyptus, lavender, linden, oak, rosemary, sunflower and sweet chestnut. In contrast Corsican honey samples contained DNA from citrus, heather, olive and rockrose more frequently than German, English and Galician honeys, although DNA from clover and rape could not been detected in Corsican honey. The lowest p-values were calculated for the olive, rape and rockrose systems. Sweet chestnut is an additional significant marker for Corsican and Galician honey compared to honey samples from Germany and England (data not shown). It was concluded therefore, that, as a proof of principle, four systems could be used to determine the geographical origin of Corsican honey. It should be noted that only 12–15 different honeys from only four countries were analysed and statistically evaluated. For more precise statements further samples, also from additional countries, should be analysed. It is very likely that honeys produced in other regions, with similar climate, would show a similar flora and therefore pollen profile. The identification of a particular plant that is only found on Corsica and the establishment of an appropriate real-time PCR detection system would have been the most promising strategy to confirm geographical origin, however, such a marker could not be identified for Corsica. Another possibility, also based on DNA methodology, would be the development of a system specific for the Corsican honey bee. Corsican honey is produced by the bee species *Apis mellifera corsica* and no other bee species have been imported onto Corsica since 1982, when a law prohibiting the importation of honeybees other than *A. mellifera corsica* came into force. Indeed, during this project, it was shown that additional DNA, other than that derived from plants, was detected in samples of honey (data not shown), where DNA from bees, aphids and bacteria were all found to be present. The identification of the Corsican honey via the identification of the species of honeybee, using genotyping, would be dependant upon the genetic drift of the Corsican honey bee since 1982, initial work on the identification of honeybee species looked promising although outside the main scope of this project.

4. Conclusions

Fifteen species specific TaqMan™ assays were developed for the detection of acacia, broom, citrus, clover, heather, eucalyptus, lavender, linden, oak, olive, rape, rockrose, rosemary, sunflower and sweet chestnut which showed no significant cross amplification when assessed on a further 10 plant species and two animal species. For the parallel detection of plants and to identify false-negative results (external amplification controls) a TaqMan™ system was developed based on a *tRNA-Leu* (trnL) sequence of approximately 90 bp. This multicopy chloroplast sequence was suitable for the detection of very low levels of plant DNA and was, therefore, applicable for the detection of plant DNA in honey. The development of a single copy *actin* sequence based system was less sensitive but may be of use for further applications, e.g. in the field of GMO or allergen analysis.

In this study, it was shown that a combination of species specific systems, selected from the pool of real-time PCR systems developed during this project, was able to produce a plant species profile unique to Corsican honey when compared to honey from Galicia, Germany and England, although the number of samples analysed was comparatively small and the locations relatively geographically remote from each other. With the detection of a particular plant in the sample that is only found in a specific location using the DNA based approach it should be possible to specifically confirm a honey's geographical origin.

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