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Pea Detection in Food and Feed Samples by a Real-Time PCR Method Based on a Specific Legumin Gene That Allows Diversity Analysis

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Real-time polymerase chain reaction is currently being used for the identification and quantification of plant and animal species as well as microorganisms in food or feed samples based on the amplification of specific sequences of low copy genes. We report here the development of a new real-time PCR method for the detection and quantification of the pea (*Pisum sativum*) based on the amplification of a specific region of the *legS* gene. The specificity was evaluated in a wide range of plant species (51 varieties of *Pisum* sp., and 32 other plant species and varieties taxonomically related or nonrelated). The method allows the detection and quantification of as low as 21.6 pg of DNA, which corresponds to 5 haploid genome copies. The system has been shown to be sensitive, reproducible and 100% specific for the rapid detection and quantification of pea DNA in processed food and feed samples, being therefore suitable for high-throughput analysis.

KEYWORDS: ***Pisum sativum*; pea; legumin; polymerase chain reaction; real-time PCR**

INTRODUCTION

Soybean is the most used and preferred protein source in animal feed due to its high protein content (44−50%) and constantly competitive price (*1*). However, the investigation of alternative protein-rich crops, including peas, beans and sweet lupins, is being encouraged for many reasons: (i) to satisfy the increased demand for high-quality protein to improve diet quality; (ii) to reduce the use of protein sources from meat and bone meal in animal diets in order to decrease the risk of the spread of bovine spongiform encephalopathy infection, as soybean consumption has also increased; (iii) to increase seed yield and protein content of the succeeding cereal crop when pulses are included in a rotation scheme (*2*). In this context, the pea could play an important role in the increasing productions of protein-rich material for animal feed as well as a protein source for human consumption as an ingredient of many food products (green peas, pasta, vegetarian meats, salads, soups and sauces, etc.) (*3*). Many studies have evaluated the nutritional interest of the pea as a beneficial component for its highly digestible protein content (19−27%), important level of essential amino acids (lysine) and low levels of antinutritive factors (*4*). It is also profitable when used in animal feed. The current use of the pea at a 15−20% rate of the full animal feed intake, replacing soybean meal or corn grain, revealed an increase of weight gain without negatively affecting ruminal fermentation and digestion (*5, 6*), and in dairy cow diets no effects on milk yield or composition have been observed (*7*). Canada, the world's largest pea producer (2,806,300 t in 2006; http://faostat.fao.org/), presented the positive effects of using peas in feed at inclusion levels of 20% broiler, 25% turkey, 20% grower−finisher pig, 30% sow, 25% cattle, 45% sheep, 30% rabbit and 15% salmonid diets (*8*). Besides, the pea represents the most important grain legume crop in Europe and is considered a solution for the increasing demand of protein-rich material as a component of animal feed, decreasing the need to import soybean (*9*).

Moreover, the commercialization of different genetically modified (GM) plant varieties is increasing rapidly, and although there are no currently transgenic peas commercially available, there are already field evaluations of transgenic varieties resistant to pea diseases or to combat infectious diseases in pigs (*10*). Thus, in the near future, the commercialization of these transgenic lines will happen and it will be necessary to comply with the mandatory rules for the labeling of by-food or feed. Therefore, for quantitative estimation of the relative amounts of GMO components in processed feed or food a species-specific endogenous control is required that estimates the total quantity of specific DNA in samples. Our work satisfies the need to obtain the above-mentioned control for the pea (*11, 12*).

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Table 1. Samples Used in the Study

	(**A**) *Pisum* sp. Species				(**B**) Other Related and Nonrelated Plant Species	
	scientific name	ITACyL AN[a]	Other AN[b]	cultivar	scientific name	common name
1	*P. abyssinicum*	ZP1237	JI0130		*Allium cepa*	onion
2	*P. abyssinicum*	ZP1246	JI1640		*Brassica napus*	rapeseed
3	*P. abyssinicum*	ZP1254	JI2202		*Brasssica oleracea*	collards
4	*P. fulvum*		JI1010		*Capsicum annuum*	sweet pepper
5	*P. sativum* subsp. *elatius* var. *pumilio*		JI0241		*Cicer arietinum*	chickpea
6	*P. sativum* subsp. *elatius* var. *pumilio*		JI1794		*Daucus carota*	carrot
7	*P. sativum* subsp. *elatius*		JI2078		*Fragaria vesca*	strawberry
8	*P. sativum* subsp. *sativum*	ZP0028		Gracia	*Glycine max*	soyabean
9	*P. sativum* subsp. *sativum*	ZP0044	JI2732	Australian Winter	*Helianthus annuus*	sunflower
10	*P. sativum* subsp. *sativum*	ZP0058		Lincoln	*Hordeum vulgare*	barley
11	*P. sativum* subsp. *sativum*	ZP0071	JI0619	Alderman	*Ipomoea batatas*	sweet potato
12	*P. sativum* subsp. *sativum*	ZP0092	JI2208	Melrose	*Lactuca sativa*	lettuce
13	*P. sativum* subsp. *sativum*	ZP0501	JI0502	Rondo	*Lathyrus cicera*	red pea
14	*P. sativum* subsp. *sativum*	ZP1010	JI0347	Kelvendon Wonder	*Lathyrus sativus*	grass pea
15	*P. sativum* subsp. *sativum*	ZP1136	JI0516	Maro	*Lens culinaris*	lentil
16	*P. sativum* subsp. *sativum*	ZP1226		Gloton	*Medicago truncatula*	medic
17	*P. sativum* subsp. *sativum*	ZP1231		Nela	*Nicotiana tabacum*	tobacco
18	*P. sativum* subsp. *sativum*	ZP1406		Coomonte	*Phaseolus vulgaris*	common bean
19	*P. sativum* subsp. *sativum*	ZP1414		Ucero	*Solanum lycopersicum*	tomato
20	*P. sativum* subsp. *sativum*	ZP1456		Cheyenne	*Solanum tuberosum*	potato
21	*P. sativum* subsp. *sativum*	ZP1457		Baccara	*Triticum aestivum*	wheat
22	*P. sativum* subsp. *sativum*	ZP1458		Iceberg	*Vicia articulata* ZU116	single-flowered vetch
23	*P. sativum* subsp. *sativum*	ZP1463		Ideal	*Vicia ervilia*	bitter vetch
24	*P. sativum* subsp. *sativum*	ZP1465		Victor	*Vicia ervilia*	bitter vetch
25	*P. sativum* subsp. *sativum*	ZP1468		Messire	*Vicia faba* var. *equina*	broad bean
26	*P. sativum* subsp. *sativum*	ZP1617		Dove	*Vicia faba* var. *mayor*	broad bean
27	*P. sativum* subsp. *sativum*	ZP1672		Blizzard	*Vicia faba* var. *minor*	broad bean
28	*P. sativum* subsp. *sativum*	ZP1677		Cherokee	*Vicia* articulata ZU116	single-flowered vetch
29	*P. sativum* subsp. *sativum*	ZP1678		Corallo	*Vicia narbonensis*	purple broad vetch
30	*P. sativum* subsp. *sativum*	ZP1683		Attika	*Vicia sativa*	common vetch
31	*P. sativum* subsp. *sativum*	ZP1687		Cartooche	*Vicia villosa*	winter vetch
32	*P. sativum* subsp. *sativum*	ZP1688	JI0617	Arthur	*Vigna radiata*	mung bean
33	*P. sativum* subsp. *sativum*	ZP1716		Paunee	*Vitis vinifera*	common grape vine
34	*P. sativum* subsp. *sativum*	ZP1717		Medora		
35	*P. sativum* subsp. *sativum*	ZP1719		Specter		
36	*P. sativum* subsp. *sativum*	ZP1720		Windham		
37	*P. sativum* subsp. *sativum*	ZP1725		Astrid		
38	*P. sativum* subsp. *sativum*			Metaxa		
39	*P. sativum* subsp. *sativum*			Shawna		
40	*P. sativum* subsp. *sativum*			Foramix		
41	*P. sativum* subsp. *sativum*	ZP4206		GP4206		
42	*P. sativum* subsp. *sativum*	PM32		PM32		
43	*P. sativum* subsp. *sativum*	PM33		PM33		
44	*P. sativum* subsp. *sativum*	ZP0104	BG1034	Pesols		
45	*P. sativum* subsp. *sativum*	ZP0109	BG1100	Titos		
46	*P. sativum* subsp. *sativum*	ZP0110	BG1121	Negrer		
47	*P. sativum* subsp. *sativum*	ZP0168		BG3033		
48	*P. sativum* subsp. *sativum*	ZP0344		Pi23		
49	*P. sativum* subsp. *sativum*	ZP0864		EslaDuero RV6		
50	*P. sativum* subsp. *sativum*	ZP1242	JI0250	*P. jomardii*		
51	*P. sativum* subsp. *sativum*		JI2605	*P. speciosum*-Libya		

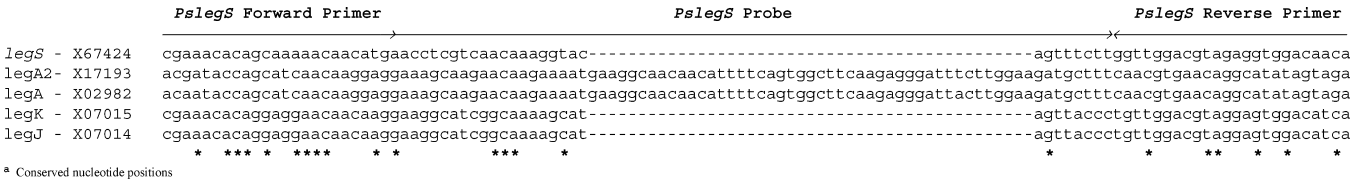
[a] AN, accession number, ZP, Germoplasm Bank of ITACyL (Instituto Tecnológico Agrario de Castilla y León); PM, Breeding Program of ITACyL. [b] JI, Germoplasm Bank of John Innes Centre; BG, Germoplasm Bank of INIA-Spain (origin Portugal).

Table 2. Quantities of *P. sativum* ZP1458 and Soy Flour Used To Prepare Experimental Feeds

pea percentage in the feed (%)	*P. sativum* ZP1458 (g)	soy flour (g)
0	0	20
0.1	0.02	19.98
0.5	0.1	19.90
1	0.2	19.80
5	1	19
15	3	17
25	5	15
50	10	10
100	20	0

The detection of plant species is mostly being performed by polymerase chain reaction (PCR), particularly by real-time PCR, which allows quantification (*13, 14*). Brezna et al. (2006) (*15*) described a method for the qualitative detection or semiquantitative determination of pea in food products based on a chloroplast gene which is in high-copy number so the achieved detection limit corresponds to less than 0.1 genome copies of pea. Therefore this method is not advisable for quantification as the copy number may not be stable within the different varieties. Furthermore, we have checked specificity of this system and found unspecific amplifications in accordance to their published record that stated unspecific amplifications over cycle 29, but particularly their system amplifies *Vicia articulata* DNA in the same manner as pea DNA, so the system is therefore not 100% pea specific.

We report the development of a real-time PCR method for the identification and quantification of the pea based on the



[a] Conserved nucleotide positions

Figure 1. Alignment of *PslegS* system amplicon in the different *legumin* genes.

Table 3. Oligonucleotides Used in This Study

target	name	type	sequence	amplicon size
rbcL gene	*RbcL*-F	forward primer	5′-TTGGCAGCATTCCGAGTAAC-3′	484 bp
	RbcL-R	reverse primer	5′-AAGTCCACCGCGAAGACATT-3′	
P. sativum legK gene	*PSlegK*-F	forward primer	5′-AAGGAGTCAAATTGTGCGAGTTG-3′	103 bp
	PSlegK-R	reverse primer	5′-TCTGTGAGAGTGAGAATGACTCTGTTC-3′	
P. sativum legJ gene	*PSlegJ*-F	forward primer	5′-CGAAACACAGCAAAAACAACATG-3′	91 bp
	PSlegJ-R	reverse primer	5′-TCCCCTTGGGTTTGATAATGC-3′	
P. sativum legS gene	*PSlegS*-F	forward primer	5′-CGAAACACAGCAAAAACAACATG-3′	75 bp
	PSlegS-R	reverse primer	5′-TGTTGTCCACCTCTACGTCCAA-3′	
	PSlegS-P	TaqMan probe	5′-FAM-ACCTCGTCAACAAAGGTACAGTTTCTT-3′TAMRA	

Table 4. Maxted and Ambrose (2001) *Pisum* sp. Classification

species	subspecies	varieties
P. sativum	*sativum*	*sativum*
		arvense
	elatius	*elatius*
		brevipedunculatum
		pumilio
P. fulvum		
P. abyssinicum		

amplification of a specific region of the *legS* gene which encodes a subunit of legumin. This hexameric protein is one of the major storage pea proteins and source of sulfur aminoacids, encoded by a minimum of 11 genes (*16, 17*). Among those genes, *legS* represented a good candidate to develop specific primers for *Pisum sativum* because it was shown to be species-specific and was conserved among the genus. The reported RTi-PCR method is therefore suitable for the detection and quantification of pea DNA for either GMO analysis or as a marker of the nutritive and protein content of processed food and feed samples.

MATERIALS AND METHODS

Materials. *Plant Species.* Eighty four different varieties from 33 plant species related and nonrelated to the *Pisum* genus were taken from the germplasm banks of ITACyL and kindly provided by the John Innes Centre (Norwich, U.K.) and the Estaçao Nacional de Melhoramento de Plantas (Elvas, Portugal) (**Table 1**). The pea population included 51 *Pisum* sp.: one *P. fulvum*, three *P. abyssinicum*, three lines of *P. sativum* subsp. *elatius* (two being var. *pumilio* and one being var. *elatius)* and 44 lines of *P. sativum* subsp. *sativum*, 33 of them being commercial cultivars and 11 landraces lines from different regions. All the samples were grown in greenhouses to collect young leaf tissue that was rapidly frozen in liquid nitrogen and stored at −80 °C until DNA isolation.

Food. Thirty nine food products which included pea among their ingredients and two foodstuffs without peas were purchased at the local market: natural peas (2), "ready to eat" food (14) (cooked peas, peas with cooked ham, peas with Spanish cured ham, pea with ham accompaniment, stewed peas with carrots, tuna and sweet pea salad, Russian salad, Mediterranean salad, beans with vegetables, lentils with vegetables, chickpeas with vegetables, pea purée with rice and hake, vegetable stew, vegetable hamburger), baby food (7) (tender peas with cooked ham, vegetables with rice, turkey with vegetables, timbale of rice with vegetables, vegetable stew, veal with vegetables, garden veal), creamed vegetables (5), dehydrated products (5) (vegetable soup, rice soup in a parsley sauce, rice with codfish and vegetables, three-jewel rice, Basque sauce), frozen meals (5) (peas, rice Milanese style, mayonnaise salad with potatoes carrots peas, vegetable salad, meat balls garden sauce) and prepared meatballs (3). The food materials were homogenized in a grinder prior to DNA extraction.

Feed. Feed was elaborated in our laboratory from soy flour and seeds of *P. sativum* ZP1458 in order to know precisely the pea concentration in the mixture. Pea seeds were powdered with a homogenizer and mixed with the soy flour to obtain the following pea percentages, expressed as grams of powered pea per 100 g of flour: 0%, 0.1%, 0.5%, 1%, 5%, 15%, 25%, 50% and 100% (**Table 2**).

DNA Isolation. DNA extraction from 100 mg of leaves of plant material was performed using the DNAeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. DNA extractions from food and feed materials were carried out from 100 mg of material using a cetyltrimethylammonium bromide (CTAB)-based method (*18*) adding a final DNA purification step using silica columns (QIAquick kit, QIAGEN). The DNA samples were eluted in 100 μL of 10 mM Tris-HCl, pH 8.0. The DNA quantity and quality were determined with the ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). DNA purified from leaves or food and feed samples was tested using a PCR system based on *RuBisCo* gene reported by Lin et al. (2000) (*24*) which amplifies a 484-fragment which is bigger than our amplicon, meaning that any amplicon smaller is susceptible to being amplified, so samples are not degraded. Moreover, we have used the commercially available 18S RTi-PCR system (Eukaryotic 18S Endogenous Control Cat. No. 4310893E, Applied Biosystems) which allowed us (i) to demonstrate that there is not even slight inhibition in the RTi PCRs and (ii) to normalize different extractions by using the C_T values obtained as a reference value to compare.

Oligonucleotides. Primers and probe (**Table 3**) were designed using the Primer Express version 2.0 software (Applied Biosystems, Foster City, CA) and synthesized by MWG-Biotech AG (Ebensburg, Germany). *Taq*Man probe was labeled on the 5′ end with the FAM (6-carboxyfluorescein) reporter dye and on the 3′ end with the TAMRA (tetra-methylcarboxyrhodamine) quencher dye.

PCR Conditions. *Conventional PCR.* Amplifications were performed in a final reaction volume of 20 μL containing GeneAmp 1× PCR Buffer II, 3 mM $MgCl_2$, 0.8 mM dNTP, 0.6 μM each primer, 0.8 U Ampli*Taq* Gold DNA Polymerase (Applied Biosystems) and 50 ng of DNA sample. PCRs were run on an Applied Biosystems 9800 Fast PCR System using the following program: 10 min at 95 °C, 40 cycles of 20 s at 95 °C, 30 s at 60 °C and 20 s at 72 °C, and a final extension of 7 min at 72 °C. Amplicons were resolved in 3% agarose gels and visualized after ethidium bromide staining.

Real-Time Quantitative PCR. PCR conditions and reagent concentrations were optimized to obtain the final parameters described below. Each reaction contained: 1× *Taq*Man Universal PCR Master Mix (Applied Biosystems), 0.3 mM of each primer, 0.15 mM of *Taq*Man

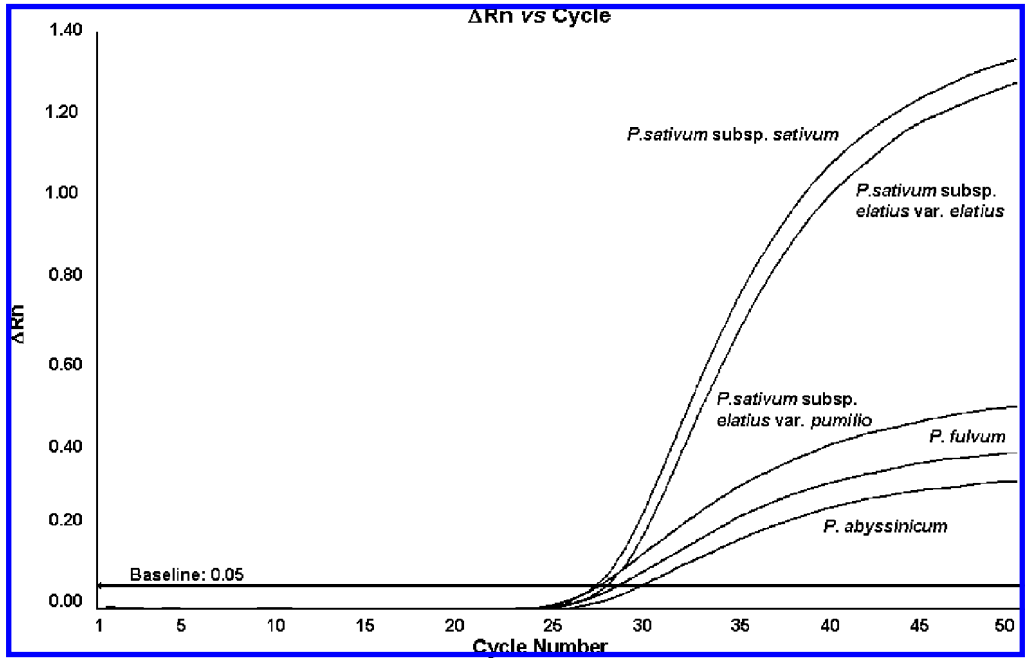


Figure 2. Amplification plot generated by different species of *Pisum* genus using the *PslegS* RTi-PCR system.

Table 5. Detection and Quantification Limits of the RTi-PCR Assay with Genomic DNA from *Pisum sativum* "Dove" Entry ZP1617

approx haploid genome copies/reaction	signal ratio[a]	C_T[b]
0.5	13/30	40.49 ± 0.27
1	16/30	40.98 ± 1.18
5	30/30	38.01 ± 0.14
10	9/9	36.99 ± 0.45
50	9/9	34.01 ± 0.21
100	9/9	33.13 ± 0.04
250	9/9	31.50 ± 0.07
500	9/9	30.44 ± 0.02
2500	9/9	28.08 ± 0.11
5000	9/9	26.95 ± 0.07

[a] Positive results in total number 9 reactions; positive results in 30 reactions for the samples that contains 5, 1 and 0.5 haploid copies. [b] C_T refers to the mean threshold cycle value ± standard error of the mean. Non Template Controls (NTC) were negative (C_T values >50 in all the replicates). These values allow to construct a standard curve described by the equation $y = -3.6686x + 40.438$, $R^2 = 0.9982$.

probe and 50 ng of DNA template, in a final volume of 20 μL. RTi-PCR reactions were run on a 7500 Real-Time PCR System platform (Applied Biosystems) using the following standard program: 2 min at 50 °C (for the AmpErase uracil N-glycosylase reaction), 10 min at 95 °C, and 50 cycles of 15 s at 95 °C and 1 min at 60 °C. RTi-PCR products were analyzed using Sequence Detection System software version 1.2.3 (Applied Biosystems). In all PCR experiments an amplification positive control (pea DNA), a Non Template Control (NTC) (soybean DNA) and a reagent control (water) have been included to test PCR performance.

RESULTS AND DISCUSSION

Selection of a Gene Fragment Suitable for Specific Detection and Quantification of Pea. Selection of suitable candidate genes was performed from public genetic sequence databases (GenBank). We looked for genes conserved within the *Pisum* genus but without homologies with other species. Among others, genes encoding storage proteins LEGUMINS were selected because of their specificity and conserved sequence within the genus. LEGUMIN constitute the major storage 11S globulins of mature pea seed (75−80%) together with the 7S vicilin. This family is encoded by a minimum of 11 genes (*19*) having heterogeneous sequence identities between 50−95% which allowed us to design the PCR system in specific and low copy regions. Initially, we chose 3 genes (*legJ*, AN X07014; *legK*, AN X07015; *legS*, AN X67424) to design three individual sets of primers (**Table 3**). The comparison of the whole sequences by using BLASTN software v2.2.14 (National Center for Biotechnology Information) showed a maximum identity apart from the respective in (i) *legJ* of 2071/2515 nt (69.6%) with *legumin* genes of *Vicia faba* and 282/2515 nt (0.09%) with *Nicotiana tabacum;* (ii) *legK* of 1305/2783 nt (46.9%) with *legumin* genes of *Vicia faba* and 478/2783 nt (17.2%) with *Lotus japonicus;* (iii) *legS* of 1674/3321 nt (50.4%) with *legumin* genes of *Vicia faba* and 966/3321 nt (29.1%) with *Glycine max*. Based on previous alignment results, primers were designed in regions which exhibit fewer similarities. Then, all PCR systems were tested experimentally in 10 species related to the *Pisum* genus by conventional PCR (*Lathyrus cicera, Lathyrus sativus, Vicia faba, Vicia sativa, Vicia articulata, Vicia villosa, Vigna radiata, Cicer arietum, Lens culinaris*), including a *Pisum sativum* sample as positive control in all the assays. Two of the PCR systems (*PslegJ* and *PslegK*) showed the presence of amplification in at least one out of 10 non-*Pisum* species tested. The system based on *legJ* gene amplified the 10 species except *Vicia villosa* and the *PslegK* system showed an unspecific band using *Lathyrus cicera* DNA. However, the *PslegS* primer set resulted totally specific to the *Pisum* genus (data not shown). Using this result as a basis, a *Taq*Man probe was also added to the *PslegS* RTi-PCR system, thus allowing the identification and further quantification. The selected *PSlegS* system gave rise to a PCR product of 75 bp for *Pisum* sp. No similarities of the amplicon were found in silico by using BLASTN software with any other plant species, nor were other legumins of pea recognized, the system being fully specific to pea and to that particular *legumin* gene (**Figure 1**).

Specificity. Pea refers to the genus *Pisum*, a member of the Fabaceae family which includes several species, subspecies and varieties. Maxted and Ambrose (2001) (*20*) proposed a modified classification from Davis (1970) (*21*) based on breeding and crossing experiments (**Table 4**). This classification divides the *Pisum* genus into three species, one of them subdivided in several subspecies and varieties. *P. fulvum* is considered the

Table 6. Detection and Quantification Limits of the RTi-PCR Pea Assay in Feed Samples

pea rate in feed (%)	pea DNA/ reaction (ng)[a]	estimated haploid genome copies/ reaction[b]	C_T[c]	calculated haploid genome copies/ reaction	relative accuracy[d]	CV[e]
0	0.00	0.00	>50	0		
0.1	0.01	1.51	38.87 ± 0.82	1.65	121.78	0.58
0.5	0.03	5.82	36.96 ± 0.73	5.84	100.18	0.76
1.0	0.06	14.17	35.89 ± 0.52	11.83	93.18	0.70
5.0	0.29	67.31	33.01 ± 0.24	79.45	103.94	0.94
15.0	0.92	212.82	31.71 ± 0.30	187.19	97.61	0.73
25.0	1.67	385.55	30.71 ± 0.22	364.05	99.04	1.46
50.0	3.22	745.64	29.58 ± 0.22	766.50	100.42	1.98
100.0	6.35	1470.14	28.48 ± 0.17	1592.65	101.10	2.11

[a] Quantity of DNA in the PCR reaction after the 50-fold dilution and 5 μL used in the mix. [b] The standard curve is described by the equation $y = -3.4814x + 39.625$, $R^2 = 0.9978$. [c] C_T refers to the mean threshold cycle value ± standard error of the mean. Non Template Controls (NTC) were negative (C_T values >50 in all the replicates). [d] Relative accuracy is defined as the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples (ratio of the Logarithm Base-10 of haploid genome copies detected by PCR and the Logarithm Base-10 of haploid genome copies estimated by measuring the absorbance of DNA by using a NanoDrop spectrophotometer multiplied by 100). [e] CV: Coefficient of variance is the ratio of the standard deviation of each C_T value to the mean of C_T values.

wild relative and it is not commonly cultivated, *P. abyssinicum* is a wild species but only cultivated in Ethiopia (*22*). Most of the current cultivated peas are *Pisum sativum* which are garden (*P. sativum* var. *sativum*) and field peas (*P. sativum* var. *arvense*) and in both cases there are worldwide commercial lines devoted to different end-uses (*23*). To satisfy the specificity criteria, the selected *PslegS* system was tested in 51 *Pisum* sp. that included 3 different species (*P. sativum*, *P. abyssinicum* and *P. fulvum*) and 28 non-*Pisum* species related and nonrelated with the genus. The amplifiability of the DNA samples was checked by conventional PCR, using a highly conservative sequence of the *ribulose-1, 5-bisphosphate carboxylase/oxygenase large* subunit (*rbcL*) gene (*24*), all samples showing positive amplification, thus being suitable for RTi-PCR assays. We defined the system 100% specific based on the negative results of all the non-*Pisum* species, and positive results of all *Pisum* sp. cultivars from different origins. The statistical analysis of the C_T *Pisum* sp. values, by ANOVA and LSD post hoc, showed no significant differences ($p < 0.05$) between the 3 species (threshold = 0.05). In this context, the system is fully specific for the *legS* gene and suitable for detection of all pea genus, regardless of the origin, the varieties, the subsp. and the sp.

Moreover, in the analysis of the specificity results, we observed different amplification patterns for the species, subspecies or varieties of *Pisum* genus assayed (**Figure 2**) which are in agreement with previous taxonomic works that reported differences between the three species of the genus (*20*). The crossing point of each amplification curve with the threshold was analyzed, and we found significant differences from cycle 30 to 36. These differences in the amplification curves could be attributed to slight polymorphisms in the sequences and allow the samples to be classified in five groups corresponding to: *P. fulvum* and *P. abbysinicum* as two species, and *P. sativum* subdivided into three groups *P. sativum* subsp. *sativum, P. sativum* subsp. *elatius* var. *elatius* and *P. sativum* subsp. *elatius* var. *pumilio*. Regarding the latest, several papers have already reported previously (*25*−*29*) that the pumilio variety shows a pattern closer to *P. fulvum* and *P. abyssinicum* but is not related to the other varieties of *P. sativum*. We have supported this classification with evidence based on a coding region of *legumin* gene which encodes a storage protein, that in legumes are clearly associated to the evolution of plant species and varieties from the wild cultivars as the *P. sativum* subsp. *elatius*. var. *pumilio* (JI1794). Therefore, the results indicate that the system offers the possibility of grouping by taxon.

Sensitivity and Quantification Accuracy of the System. *Pisum* sp. has an approximately 4,172 Mbp/1C genome (which weigh 4.32 pg) stable in size between species (*30, 31*). To test the sensitivity of the *Psleg*S system, serial dilutions of *P. sativum* genomic DNA ZP1617 ranging between 21.6 ng and 2.16 pg, which corresponded to 5,000, 2,500, 500, 250, 100, 50, 10, 5, 1 and 0.5 haploid copies, were analyzed. The assay was carried out by three independent experiments which included 9 replicates for each dilution, except the dilutions that contained 5, 1 and 0.5 haploid copies, which were assayed 30-fold. The results (**Table 5**) allowed us to construct a standard curve defined by the equation $y = -3.6686x + 40.438$, with a regression coefficient close to 1 ($R^2 = 0.9982$), which indicates a highly linear assay and a system efficiency of 0.873, which is consistent with other previously published work (*32, 33*). The detection limit was defined as the minimum level at which the analyte can reliably be detected with a probability of 95% (*34*), being in our system 5 haploid genome copies per reaction, which corresponds to 21.6 pg of pea genome. On the other hand, the quantification limit is the lowest concentration at which there is some confidence of the accuracy of the reported measurement (*35*). Based on this definition and the variance analysis, ANOVA, LSD (least significant difference), Duncan and Student−Newman−Keuls, post hoc analysis, we did not find C_T overlaps in the range from 5000 to 5 haploid copies and we determined that the quantification limit is also 5 haploid copies.

Implementation of the Assay to Processed Samples. *Food.* Thirty-nine processed food products containing pea within the ingredients and two more samples containing other vegetables but not peas were analyzed with the *PslegS* system to detect the presence of pea. The system was able to detect the presence of pea DNA in 38 samples, only one meatball sample out of 3 samples tested showed no amplification and neither did the food containing other vegetables (2 samples) but no pea.

Feed. Animal feed was prepared with different percentages of pea and analyzed to determine the accuracy of the system applied to feed analysis. The DNA samples obtained were diluted 50-fold and 5 μL was used as DNA template in the PCR. Two PCR assays were made individually. In each experiment all the percentages were tested 5-fold (**Table 6**). The variance analysis of the C_T from different feed and post hoc tests defined the detection limit of pea in feed at 21.6 ng of pea genomic DNA in the reaction, so the system allows quantification as low as 0.5% of pea in animal feed and, furthermore, allows pea traces to be detected in animal feed with a detection probability of

70%. All the percentages analyzed presented significant differences, and the homogeneous subsets distributed all the percentages in different groups according to the results. A non-pea feed (0%) was simultaneously analyzed as negative control obtaining a $C_T > 50$, demonstrating the absence of unspecific amplifications. Thus, the *PslegS* system was shown to work in the range of optimal percentages suggested in several reported feed studies for different animals (poultry, cattle, pigs or fish).

In conclusion, a *Pisum* sp. real-time PCR system has been designed and shown to be specific for the *Pisum* genus, and it does not amplify any other related or nonrelated plant species. The sensitivity of the system has been tested, and the post hoc analysis determined a limit of detection and quantification of 5 haploid copies (21.6 pg) per PCR reaction. In this sense, the system allows the presence of pea to be detected and quantified in the percentages used in feed, and moreover, we have shown that the system allows the presence of traces to be detected in both food and feed materials. The *PslegS* RTi-PCR is a reproducible and fully specific method for the detection and quantification of pea in processed food and feed samples, which can be carried out in a short time and being suitable for high-throughput analysis. This reported system is therefore available for further reduction to nanolitre quantities to be used in miniaturized platforms as "lab-on-a-chip" analysis that could be easily implemented in routine diagnosis laboratories.

ABBREVIATIONS USED

%, percentage; μL, microliters; μM, micromolar; AN, accession number; ANOVA, analysis of variance; BLASTN, Nucleotide Basic Local Alignment Search Tool (NCBI); C_T, threshold cycle; *E*, efficiency; F, forward; FAM, 6′-carboxyfluorescein; GMO, genetically modified organism; g, grams; LSD, least significant difference; *M*, mean values; Mbp, million base pairs; mg, milligrams; min, minutes; mM, millimolar; ng, nanograms; °C, degrees Celsius; P, probe; PCR, polymerase chain reaction; pg, picograms; R, reverse; R^2, regression coefficient; RTi-PCR, real time polymerase chain reaction; s, seconds; SD, standard deviation; TAMRA, tetramethylcarboxyrhodamine.

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