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ORIGINAL PAPER

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Polymerase chain reaction (PCR) for the detection of king bolete (*Boletus edulis*) and slippery jack (*Suillus luteus*) in food samples

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Abstract Expensive food is always a possible target for fraudulent labelling. King bolete (*Boletus edulis*) belongs to the most popular and expensive mushrooms in Europe. Polymerase chain reaction (PCR) analysis on the internal transcribed spacer (ITS) is a suitable method of identifying mushroom species. The ITS region of several *Boletus edulis* and several closely related mushroom species (e.g. *Suillus luteus*) was sequenced. Using these results specific PCR methods could be established. Furthermore it was shown that a mushroom sold as king bolete originated in China, and is actually another mushroom species. A market survey showed that in highly processed food products with labelling identifying king bolete in fact always contained these Chinese bolete species.

Keywords Basidiomycetes · Boletus · Suillus · Polymerase chain reaction (PCR) · Mushroom identification

Introduction

Mushrooms are popular in many dishes, especially for their flavour. Edible mushrooms are suitable as ingredients for soups, sauces, pies, patés, risotto and pasta products. From the wild grown mushrooms the use of king bolete (*Boletus edulis*) has received a certain importance in European culture. Less important are other mushroom species of the same genus as the summer bolete (*Boletus aestivalis*), the pine bolete (*Boletus pinicola*) or the black bolete (*Boletus aereus*). Food with a portion of these mushrooms are especially labelled with the characterisation “bolete-”.

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Many tons of mushrooms per year were imported into Switzerland mainly from China, the Balkan States, Germany and Italy. Among imported forest mushrooms the amount of slippery jack (*Suillus luteus*) and *Suillus collinitus* has been considerable. From the genus *Suillus* in 1999 more than 12 tons of dried and often granulated mushrooms were imported.

When the method was first developed for the detection of mushrooms of the genus *Suillus*, *Suillus* was never mentioned in food labelling. This, together with the fact that 12 tons per year were being imported, made us developing a method for the detection of fraudulent addition of the cheaper slippery jack to the more expensive king bolete. The genera *Boletus* and *Suillus* both belong within the class *Basidiomycetes* of the order *Boletales*. Therefore *Boletus* and *Suillus* are phenotypically closely related. Still it is not difficult to distinguish the two genera in whole mushrooms. Very often microscopic examination leads to the mushroom species, but in highly processed products as dried soup, patés or pies, microscopic examination is not applicable. DNA-based methods have been used in the last few years to identify all kinds of mushroom species. The PCR followed by a restriction fragment length analysis or a sequencing has been shown to work for the identification of meat species [1, 2, 3], fish species [4, 5, 6] and plant species [7, 8].

DNA analysis in fungi was mostly used for phylogenetic studies [9, 10]. However, using PCR for the identification of fraudulent addition of mushrooms was already being done in truffles [11, 12, 13]. The isolation of DNA from fruit bodies and from mycorrhizal roots was described by Fischer et al. [10, 14] for the phylogenetic examination of the *Boletales*. In fungi PCR-amplification using the conserved primer sequences ITS1 and ITS4 was successfully used to amplify a partly variable, multiply present region of the internal transcribed spacer (ITS) [15]. With this it is a question of nucleus coded rDNA-fragments in between the 18S and 5.8S rRNA-genes, 5.8S and 28S rRNA-genes respectively. The primer pairs ITS1 and ITS4 gives amplicons with DNA from different edible mushrooms of a length in between 700

and 1000 basepairs. A restriction fragment length polymorphism analysis allows the identification of the fungi species (data not shown). If one analyses processed food products where, besides pure mushroom, other ingredients like tomatoes (*Lycopersicon esculentum*), maize (*Zea mays*), rice (*Oryza sativa*) or carrots (*Daucus carota*) are amplified, the resulting restriction enzyme patterns are no longer readable.

In this work methods for the detection of *Boletus* and *Suillus* are described to allow an identification of fraudulent addition of cheaper *Suillus* species to food products labelled with the popular and more expensive *Boletus* species. While working on these methods it was shown that the mushrooms used in processed food labelled as king bolete (*Boletus edulis*) originated from China and thus have to be a different mushroom species or subspecies and not the European king bolete. Subsequently a method that allows differentiation of this so far unknown species from king bolete is described. This system can be used together with the *Boletus*- or *Suillus*-specific methods in a multiplex assay that allows the detection of an addition of less than 5% of the "Chinese" bolete.

Materials and methods

Reference species. Reference fungi samples were mostly collected and authenticated by a federal fungi controller. Where the species was easy to identify and commercially available (cultivated mushroom, chanterelle) reference material was bought in food stores. Different food products containing mushroom were also bought in food stores. The investigated fungi are listed in Table 1.

Isolation of nucleic acids. The reference fungi were dried overnight in an oven at 50 °C and homogenised with mortar and pestle. The mushroom pieces in dry food products were separated using tweezers and homogenised as above; wet material was homogenised using a Polytron mixer. Then 50 mg of dry sample plus 100 µl of deionised water, or 300 mg of fresh sample, was weighed into a sterile 2-ml Eppendorf tube. To each sample 700 µl extraction buffer [50 mmol/l Tris-HCl (pH 7.2), 50 mmol/l EDTA, 3% (w/v) sodium dodecyl sulphate, 0.1% 2-mercaptoethanol] was added and mixed by vortexing. The mixture was incubated for 1 h at 65 °C with shaking. The lysate was cooled to room temperature, then 10 µl of 10 mg/ml RNAse A (Qiagen, Hilden, Germany) was added and incubated at room temperature for 3 min. To each sample 600 µl of phenol:chloroform:isoamyl alcohol, 25:24:1 (Sigma, St Louis, Mo) was added and vortexed. The mixture was centrifuged for 15 min at 10,000 g. The aqueous supernatant was transferred to a sterile 1.5-ml Eppendorf tube and an ethanol precipitation was done by adding 50 µl of 3 mol/l sodium acetate (Merck, Darmstadt, Germany) and 350 µl isopropanol (Merck) followed by gentle mixing. After centrifugation for 2 min at 15,000 g the pellet was washed with 200 µl of 70% ethanol (Merck) and re-centrifuged for 1 min at 15,000 g. This washing step was repeated once. After drying in a vacuum desiccator for 10 min, the DNA pellet was resuspended in 200 µl of elution buffer [10 mmol/l Tris (pH 9.0)] preheated to 70 °C, and stored at –20 °C.

DNA-concentrations were determined by $UV_{260\,nm}$ spectrophotometry (Lambda 12; Perkin Elmer, Branchburg, N.J.) according to the Swiss Food Manual [16].

Polymerase chain reaction. DNA amplification was carried out in a final volume of 100 µl in 0.5-ml thin wall tubes (Witec, Littau, Switzerland). Each reaction contained 1× reaction buffer (Promega, Madison, Wis.), 2.5 mmol/l magnesium chloride (Promega), 2.0 µg/ml bovine serum albumin (Pharmacia, Uppsala, Sweden), 0.2 mmol/l of each 2′-deoxynucleoside-5′ triphosphate (Sigma), 0.5 µmol/l of the primers (sequences are listed in Table 3) ITS1-F and ITS4-B, ITS1-F and BED-4, ITS1-F and BED-2 or ITS1-F and SLU-1 (Microsynth, Balgach, Switzerland) respectively, and 2 units of *Taq* DNA polymerase (Promega). PCR was performed in a Progene thermocycler (Techne, Princeton, N.J.) with the following temperature program: denaturation at 95 °C for 3 min, 35 cycles at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 80 s and a final extension step at 72 °C for 3 min.

Multiplex PCR. The multiplex PCR was carried out with the same concentrations and temperature program as described above except for the following adaptations: the magnesium chloride concentration was increased from 2.5 mmol/l to 3.5 mmol/l. The concentration of the forward primer ITS1-F was doubled to 1.0 µmol/l and the reverse primer BED-2 was used less concentrated (0.16 µmol/l). The second reverse primer SLU-1 was used as described above (0.5 µmol/l).

Gel electrophoresis. PCR products were separated on a 2% agarose gel (Roth, Karlsruhe, Germany) in 1× TBE buffer [50 mmol/l Tris, 50 mmol/l boric acid, 1 mmol/l EDTA (pH 8)] stained with ethidium bromide. As size standard a 100-bp (base pair) ladder (Pharmacia) was used.

DNA-sequencing. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) corresponding to the producer manual. The purified PCR products were sequenced on a DNA sequencer (ABI Prism 377, Perkin-Elmer) using fluorescence dye labelled dideoxynucleotides (Microsynth, Balgach, Switzerland).

Sequence comparison of ITS PCR fragments against each other. DNA sequences received from the sequencing experiments were used to compare the nuclear rRNA ITS gene region of different mushrooms for the primer design of genus-specific PCR-systems. For that purpose the PCR fragments were compared to each other

Table 1 Investigated *Basidiomycetes* species

Species (*Latin name*)	Species (English)	Species (German)	Family
Boletus edulis	King bolete	Steinpilz	Boletaceae
Boletus aestivalis	Summer bolete	Sommer-Steinpilz	Boletaceae
Boletus erythropus	Dotted-stemmed bolete	Flockenstieliger Hexenröhrling	Boletaceae
Suillus luteus	Slippery jack	Butterpilz	Boletaceae
Suillus grevillei	Larch bolete	Goldröhrling	Boletaceae
Suillus collinitus	unknown	Ringloser Butterpilz	Boletaceae
Xerocomus badius	Bay boletus	Maronenröhrling	Boletaceae
Xerocomus chrysenteron	Red-cracked boletus	Rotfussröhrling	Boletaceae
Agaricus bisporus	Cultivated mushroom	Kultur-Champignon	Agaricaceae
Cantharellus cibarius	Chanterelle	Eierschwamm	Cantharellaceae

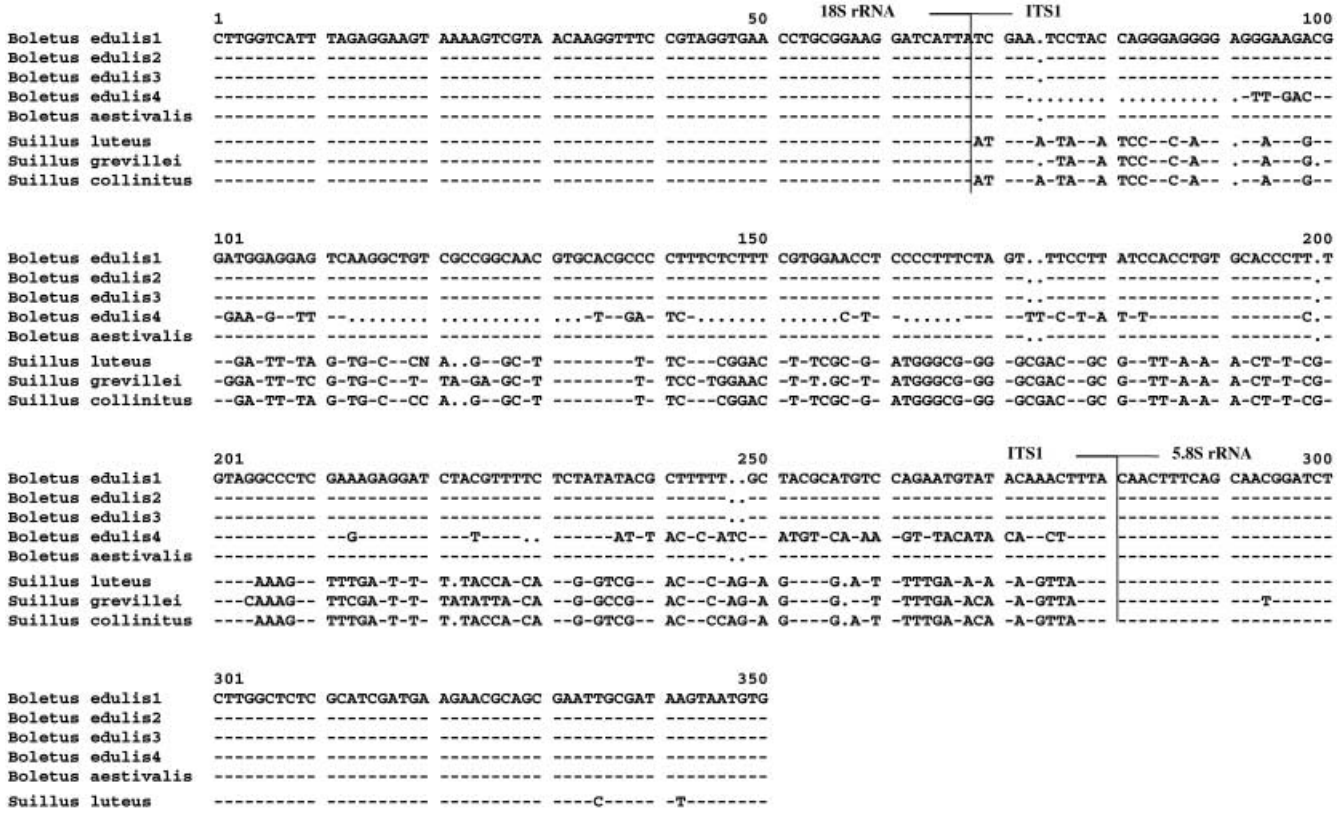

Fig. 1 Sequence alignment. The sequence of *Boletus edulis* 1 is completely listed. "-" symbolizes the same basepair as *B. edulis*. "." stands for the lack of a basepair. *B. edulis* 1 and 2 were collected in Switzerland. *B. edulis* 3 has its origin in Austria and *B. edulis* 4 in China, respectively

with the "Multiple sequence alignment with hierarchical clustering"-function from the online software programme "Multalin version 5.4.1" (http://www.toulouse.inra.fr/multalin) [17]. To perform the alignments the symbol comparison table "blosum62" was chosen.

Results and discussion

Sequencing

The sequence information of the nuclear rRNA ITS gene region for edible mushrooms is at present mostly unavailable in databases. For most *Boletus* and *Suillus* species no entries could be found in the sequence database. Therefore the PCR amplicons of the rRNA ITS gene region produced with the basidiomycete specific primers ITS1-F and ITS4-B were purified and sequenced. PCR products vary in size between 700 and 1000 bp (data not shown). Figure 1 shows a sequence alignment of a 350 bp long part of this gene region consisting of 18S rRNA, ITS1 and 5.8S rRNA of the investigated species of the genus *Boletus* and *Suillus*. As expected there is almost no difference between all compared *Boletaceae* sequences in the 18S rRNA and the 5.8S rRNA region. The ITS1 region of the *Boletus* and the *Suillus* genus shows only little homology. Three samples of European king bolete (*Boletus edulis*) with different origins and the summer bolete (*Boletus aestivalis*) show 100% identity. The sequence from the king bolete of Chinese origin is different to the other *Boletus* species. There is above all a lack of 61 bp which is symbolized with dots in Fig. 1.

Sequence comparison

In Table 2 the degree of relationship of different mushroom species within their 18S rRNA-ITS1 PCR fragments is stated in %. The three samples of European king bolete and the summer bolete (*boletus aestivalis*) show 100% identity. The ITS1 sequence of "Chinese king bolete" is 61 bp shorter than the ITS1 sequence from the other *Boletus* species. The homology without the deleted 61 bp is 80% (232 from 289 bp). The total homology ends up at only 68% agreement. These results shows that the "Chinese king bolete" is a species in its own right, which probably belongs to the family *Boletaceae* but is not identical to the European king bolete (*Boletus edulis*).

The investigated *Suillus* species which do all belong to the family *Boletaceae* show 91–99% homology to each other, which demonstrates their close relationship. The comparison between *Suillus* and *Boletus* species results in 54–62% agreement. In the conserved 18S and 5.8S rRNA gene region both *Boletus* and *Suillus* species show nearly 100% identity. *Boletus* and *Suillus* only differ in their ITS1 gene region. *Xerocomus chrysenteron* also belongs

Table 2 Degree of relationship within the 18S rRNA-ITS1 PCR fragment of investigated mushrooms (as percentages)

Scientific name	*Boletus edulis* (Eu)	*Boletus edulis* (Ch)	*Boletus aestivalis*	*Suillus luteus*	*Suillus grevillei*	*Suillus collinitus*	*Xerocomus chrysenteron*	EMBL-ID-Nr.	English name
Boletus edulis (Europe)	100							AJ416954	King bolete
Boletus edulis (China)	68	100						AJ416955	King bolete
Boletus aestivalis	100	68	100					AJ416956	Summer bolete
Suillus luteus	55	57	55	100				AJ416957	Slippery jack
Suillus grevillei	56	62	56	91	100			AJ416958	Larch bolete
Suillus collinitus	54	57	54	99	91	100		AJ416959	Unknown
Xerocomus chrysenteron	67	71	67	63	62	63	100	AJ416960	Red-cracked boletus

Table 3 Oligonucleotides used for PCR

Name	Sequence (5′–3′)	Reference	Position in Fig. 1
ITS1-F	5′-CTT GGT CAT TTA GAG GAA GTA A-3′	[19]	1–22
ITS4-B	5′-CAG GAG ACT TGT ACA CGG TCC AG-3′	[19]	Not shown in Fig. 1
BED-2	5′-ACG TTC TGG ACA TGC GAT AGA G-3′	This study	242–263
BED-4	5′-GTT TGT ATA CAT TCT GGA CAT GCG-3′	This study	253–275
SLU-1	5′-ACT TTT TTC TCA AAG AAT CGC GTC-3′	This study	251–275

to the family *Boletaceae* and the homology to the *Boletus* species is between 67 and 71% compared to a homology between 62 and 63% to the *Suillus* species. The sequences from *Agaricus bisporus* and *Cantharellus cibarius* are too different from the *Boletaceae* species to apply a reasonable sequence comparison.

System setup

The sequence information was used to develop specific primers for the detection of *Suillus luteus* and *Boletus edulis*. For an efficient control it is important to have a system that detects highly processed food in which the DNA is sometimes highly fragmented [18]. For this reason the amplified fragments should be as small as possible. An ideal detection method for the food control should not be too specific. If a method detects only a single fungi species, such as for example *Boletus edulis*, the possibility of applying the method would be too limited. The goal was the development of a method that is specific for the genus of interest (*Boletus* or *Suillus*, respectively). The sequence alignment (Fig. 1) resulted only in little regions with enough homology for the primer positioning, that also excludes a specificity to another genus. As forward primer the ITS1-F basidiomycete primer located in the highly conserved 18S rRNA was taken, as described by Gardes and Bruns [19]. The reverse primers were set specifically in the variable ITS1 rRNA gene region for *Boletus* or *Suillus* (Table 3).

The PCR using the *Boletus*-specific primer BED-4 produces fragments of 270 bp and 209 bp for European king bolete and “Chinese king bolete”, respectively. A second *Boletus*-primer BED-2 amplifies specifically “Chinese king bolete” with a fragment size of 201 bp. The *Suillus*-specific primer SLU-1 results in a PCR product of 270 bp.

Specificity

Cross reactivity between the genera *Boletus* and *Suillus* was tested with the available fungi species. Both PCR systems are specific for their corresponding genus. Fungi of other families often used in food and food products have been tested as well, without showing any cross reactivity with the described systems. Reference fungi of the genus *Xerocomus* which are hardly used in commercial food products showed cross reaction with the *Boletus*-specific system with primer BED-4. With the primers BED-2 and SLU-1 no cross reactivity was detected. The result of the specificity testing is listed in Table 4.

Sensitivity

Using tenfold dilutions of DNA extracted from *Boletus edulis* and *Suillus luteus* the sensitivities of the systems were determined. Because of the rather small genome size of mushrooms (around 100 times smaller than the genome size of maize or soybeans) [20], the expected sensitivity is rather high. Expecting a haploid genome size of 5×10^7 bp the expected sensitivity is at least 0.02 ng [21]. Starting with a DNA-concentration of 200 ng tenfold dilutions were detected with the described PCR systems showing that the expected sensitivity of 0.02 ng could be achieved (data not shown). Therefore if we test a food sample containing around 5% fungi, the detection of a 1% addition of *Suillus luteus* in a *Boletus edulis* product (corresponding to 0.1 ng DNA from *Suillus luteus*) can still be detected.

Table 4 Specificity of the PCR-detection systems

Species (*Latin name*)	*Boletus*-system with primer BED-2	*Boletus*-system with primer BED-4	*Suillus*-system with primer SLU-1
Boleutus edulis (China)	+	+	–
Boletus edulis (Europe)	–	+	–
Boletus aestivalis	–	+	–
Boletus erythropus	–	–	–
Suillus luteus	–	–	+
Suillus grevillei	–	–	+
Suillus collinitus	–	–	+
Xerocomus badius	–	+	–
Xerocomus chrysenteron	–	+	–
Agaricus bisporus	–	–	–
Cantharellus cibarius	–	–	–

+ = PCR-signal was detected;
– = no PCR-signal was detected

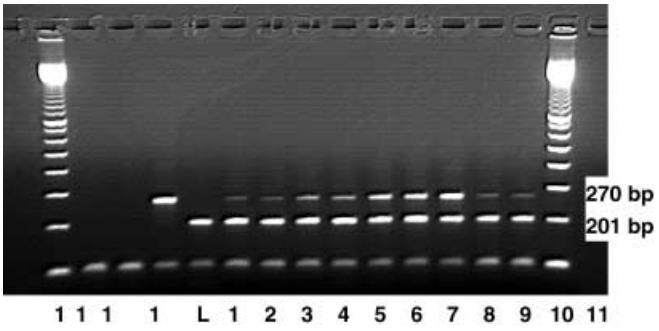

Fig. 2 Multiplex of different dilutions of *Suillus* and Chinese-"bolete" using the primers ITS1-F/BED-2/SLU-1. The size of the *Suillus luteus* and the Chinese-"bolete" PCR were 270 and 201 bp, respectively. Lane L 100 bp ladder; lane 1: mastermix control; lane 2: DNA from *B. edulis*; lane 3: DNA from *Suillus luteus*; lane 4: DNA from the Chinese-"bolete"; lanes 5,6: 1% *S. luteus*/99% Chinese bolete; lanes 7,8: 3% *S. luteus*/97% Chinese bolete; lanes 9,10: 10% *S. luteus*/90% Chinese bolete; lane 11: 50% *S. luteus*/50% Chinese bolete; lanes 12,13: sample A

Multiplex PCR

With a multiplex PCR that uses the primers BED-2 and SLU-1, two fragments of 201 bp and 270 bp are produced for "Chinese king bolete" and *Suillus* species, respectively. The two PCR systems (primers BED-2 and SLU-1) use the same forward primer ITS1-F and produce fragments of a length of 201 bp and 270 bp. The multiplex system using the primers ITS1-F, BED-2 and SLU-1 needed some adaptation of primer concentration and magnesium chloride, but then the system showed robust results (Fig. 2). In the multiplex system a loss of sensitivity was observed. Still an addition of less than 5% *Suillus* or Chinese king bolete was easily detected and the multiplex assay even allows a semi-quantitative estimation. In Fig. 2 different mixtures of slippery jack and Chinese king bolete were analysed. Sample A is a mushroom soup. Using the multiplex assay it was possible to estimate the amount of slippery jack, which is between 1 and 3%. The limitation of this multiplex system is the fact that it cannot detect *Boletus edulis*. Still this multiplex system is an excellent way to detect the fraudulent addition of *Suillus* as well as "Chinese king bolete" to food products labelled with the ingredient *Boletus edulis*. The presence of *Boletus edulis* can always be checked using the BED-4 Boletus PCR-system.

Table 5 Results of the market survey

Food product	*Boletus* labelled/found	*Suillus* labelled/found
Mushroom sauces	7/7	2/2
Soups	9/9	3/2
Mushroom risotto	6/6	3/3
Pasta with mushroom	6/6	2/2
Patés or pies	1/1	
Semolina with boletus	1/1	
TOTAL	30/30	10/9

Application of the method on different food matrices

During spring 2001 30 food samples with a labelling of king bolete were tested as to whether there were "Chinese king bolete" or slippery jack present. The 30 samples included products like sauces, bouillons, pastas, risotto, etc. (Table 5). An identification of the mushroom species was mostly impossible with classical methods because of former technological procedures (homogenisation, temperature, etc.). Still, in all samples DNA could be extracted and the PCR-determination of the mushroom species worked well. In 10 of the 30 samples slippery jack was labelled as an ingredient. This was surprisingly different to the situation two years ago where no food samples could be found with a labelling of slippery jack on the Swiss market. The addition of slippery jack could be confirmed in 9 of 10 samples. The detection of king bolete resulted in an unexpected result. In all 30 samples the presence of the "Chinese king bolete" was detected. The mushroom that is sold as king bolete from China is in reality not a king bolete.

Conclusion

The systems described allow detection of very small amounts of *Suillus* or *Boletus* respectively. If we use the system as a multiplex assay it is still possible to detect

fraudulent additions of at least 1–2%. As we allow the presence of around 2–5% of non-labelled mushroom the multiplex detection system is sensitive enough for the quality control. The multiplex method saves time and consumables. The results of the multiplex system is even semi-quantitative and allows an estimation of the added amount of a mushroom (see Fig. 2). In processed food the use of "Chinese king bolete" seems to be common. It has to be checked in what relationship the "Chinese king bolete" stands to king bolete. Corresponding to the degree of relationship between *Boletus edulis* and the "Chinese king bolete", the mushroom sold as king bolete originated in China can hardly be the same species. Jarosch and Bresinsky [22] have formulated a similar suspicion.

The system setup could be adapted for the development of other mushroom species as long as it refers to *basidiomycetes*. For *ascomycetes* other PCR systems to produce the necessary sequence information need to be used.

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