Authentication of commercial candy ingredients using DNA PCR-cloning methodology

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Abstract

BACKGROUND: Commercial candies are consumed by all population age sectors worldwide, thus methods for quality control and composition authentication are needed for best compliance of consumer's preferences. In this study the applications of DNA-based methodology for candy quality control have been tested. Eighteen samples of commercial candies (marshmallows, gumdrops, jelly, sherbet, gelatin-based desserts) produced by five countries were analyzed to identify the component species by Polymerase chain reaction, cloning and sequencing of 16S rRNA and ribulose -1,5-diphosphate carboxylase oxygenase genes, and the species determined from BLAST comparison with universal databases and phylogenetic analysis.

RESULTS: Positive DNA extraction and amplification of the target genes was obtained for 94% of candies assayed, even those containing as little as <0.0005 ng/µl DNA concentration. The results demonstrated that the species detected from DNA were compatible with the information provided on candy labels only in a few products. DNA traces of undeclared species, including fish, were found in most samples, and two products were labeled as vegetarian but contained porcine DNA.

CONCLUSION: Based on the inaccuracy found on the labels of sweets we recommend the use of DNA tests for quality control of these popular sweets. The DNA-tests have been useful in this field but Next Generation Sequencing methods could be more effective.

Keywords: Candy products, DNA tests, vegetarian labeling, consumer's choice, traceability, labels.

INTRODUCTION

Candies are consumed worldwide in different cultures and countries.[1] The use of sweets in human diet is very old: ancient Arabian, Chinese and Egyptian peoples consumed candied fruits and nuts cooked with honey, and the Aztecs made a chocolate drink with cocoa seeds.[2] Now candies are consumed by all population sectors and ages; since they are especially popular for children,[3,4] their quality control should be a priority.

The composition of commercial candies and sweets is complex and often includes many food additives and preservatives.[5] Many products like soft and jelly-based candy contain gelatin, which is frequently made from pig and cow.[6,7] The presence of these animals in candy may raise ethical or religious issues in some consumer sectors, for example in vegetarians and in Halal-Kosher consumers,[7] and consumers should be informed about their choice. Information about the ingredients is important for consumer's health also, since adverse reactions have been reported for allergic patients who consumed candies without knowing they contained saffron,[8,9] cochineal-made carmine[9] and peanuts.[10] Anaphylactic shocks after eating marshmallows made from undisclosed fish gelatin have been also documented.[11] Therefore, disclosing full information about the species contained in commercial candies is essential for consumers to know what they are eating and help them to make ethical and safe choices.

Determining the species composition in commercial candies is not easy because they are generally highly processed and can contain a mixture of products. DNA has been often used for determining species composition in food, and nowadays the techniques for DNA extraction and amplification by Polymerase chain reaction (PCR) allow the successful analysis of highly processed products,[12-15] detecting even small traces.[16] In the present study we have purchased different types of candies produced by five

countries, for determining their species composition employing DNA-based molecular techniques. Two different primer sets specific for animal or plant species were employed for PCR amplification and cloning of DNA extracted from the candies. From the results we have assessed the utility of this DNA-based methodology for quality control in candy markets. The following parameters were considered for the assessment: DNA content, PCR-amplification success, number of species detected, accuracy of species identification (from concordance of two assignment methods, BLAST-based and phylogenetic). In addition, comparing the detected and declared species we have evaluated the accuracy of current candy labelling to recommend improvements in quality control, if needed.

EXPERIMENTAL

Sampling

Eighteen candies from five countries: Spain (8 samples), France (2 samples), Portugal (4 samples), Sweden (1 sample), Spain/Portugal (2 samples), Spain/Turkey (1 sample) were analyzed (Table 1). Different products were considered (Fig. 1), including raw gelatin powder (3), desserts (3), soft candies (7), marshmallows (2), gums (2) and sherbet powder (1). The information provided in each candy label was analyzed in detail, especially the list of ingredients, allergy warnings and indications for specific consumer sectors such as vegetarians, vegans and persons with food restrictions.

DNA extraction, quantification, PCR amplification and sequencing process

DNA analysis was carried out in strict sterile conditions to prevent contamination, and both pre- and post-PCR processes were controlled. Sample manipulation was done within a sterile room cleaned with 100% ethanol and 10% bleach. All the material employed was cleaned and put in sterile bags to autoclaving. DNA extraction and PCR

amplification were performed into a flow chamber within that sterilized room, with ultraviolet light to ensure destruction of any possible contaminant DNA. During all the process researchers wore two pairs of gloves, paper mask and cap and laboratory coat. Negative controls were used to check possible contamination during the laboratory analysis, from the DNA extraction process to the visualization of PCR products in agarose gels. Pig (*Sus scrofa*) and rainbow trout (*Oncorhynchus mykiss*) were used as positive controls of animal detection with the 16S rRNA gene, and apple (*Malus domestica*) was the positive control for plant detection with rbcL gene. For the rest of post-PCR processes we worked in other laboratory within another flow chamber, also under ultraviolet light.

DNA extraction was performed with the kit DNeasy Mericon Food Kit of QIAGEN. From each candy four Eppendorf tubes with 200 mg each one were employed for DNA extraction to get more amount of DNA. Two cleaning steps were done to eliminate potential inhibitors of polymerase chain reaction: first with CTAB detergent and second with chloroform. After, the contents from the four tubes of the same candy was put together to continue with the rest of the protocol. DNA was quantified using Qubit dsDNA HS Assay Kit in a Qubit 2.0 Fluorometer. The detection limit of this method is 0.0005ng/µl.

PCR amplification was done with the kit PCR core Kit Plus of Roche, with the enzyme uracil glycosylase and dideoxynucleotide with uracil instead of thymine. Since candies are highly processed their DNA is likely degraded,[17] therefore we targeted short species-specific sequences. For animal species we employed the primers 16S-HF 5'-ATAACACGAGAAGACCCT-3'and 16S-HR 5'-CCCRCGGTCGCCCCAAC-3' developed by Horreo *et al.*[18] that amplify an 80-122 base pair (bp) fragment within the 16S rRNA gene. PCR reaction was performed with: 5 µl of DNA extraction from the

candy (from less than 0.0005 ng/µl to 0.328 ng/µl), 0.125 µl of Taq polymerase from the PCR core Kit Plus of Roche, polymerase solution with Mg^{2+} 1x, 0.5 µl of each primer 10 µM, 0.5 µl dideoxynucleotides (dNTPs with U), 0.5 µl of Uracil glycosylase and bi-distilled water up to 25 µl of total volume. The PCR conditions were: a cycle at 20 ºC for 5 min and at 95ºC for 2 min to activate and deactivate the uracil glycosylase enzyme respectively; then, 40 cycles at 95ºC for 30s, 55ºC 30s, 72ºC 1min 30s, and a final cycle at 72ºC during 7 min. For plant species we employed the primers Plant159-F CTTGATTTTACCAAAGATGATGA and Plant159-R TTCTTCGCATGTACCCGCAG designed by Han *et al.*[19] for amplifying a 159 bp fragment of the ribulose-1,5-diphosphate carboxylase oxygenase gene (rbcL). PCR reaction was performed in the same way than 16S rDNA gene but the PCR conditions after the cycle for uracil glycosylase enzyme were 50 cycles at 95ºC for 30s, 58ºC 30s, 72ºC 1min 30s, and a final cycle at 72ºC during 7 min.

PCR products were run in 2% agarose gels stained with ethidium bromide. Purification of PCR product was performed with IllustraIM ExostarIM 1-Step de GE Healthcare Life Sciences. Direct sequencing was performed at the sequencing facilities of the University of Oviedo employing BigDye Terminator Cycle Sequencing chemistry and ABI Prism 3130xl Genetic Analyzer. Chromatograms evidenced species mixture, thus cloning approaches were employed to obtain individual sequences. After purification of PCR product with Wizard SV Gel and PCR Clean-Up System Kit (Promega), the purified DNA was cloned using the Dual Promoter TA Cloning Kit (Invitrogen), with pCR II vector and competent cells TOP10'. Briefly, we did the ligation and performed a transformation process by thermal shock. Then the bacteria (*Escherichia coli*) were grown in liquid SOC medium for 1 hour and spread on solid LB medium with ampicillin. When bacteria grew (only bacteria carrying the vector are ampicillin-

resistant) we picked white colonies, which carry the insert (their β-galactosidase gene is interrupted) in 50 µl of bidistilled water. DNA was extracted from the colonies by thermal shock at 95 ºC during 5 min and a PCR was performed using the primers T7 and SP6 located in the flanking regions of the insertion site. The reaction mix of this PCR was: 1.5 units of Taq polymerase of Biotools (5U/µl), polymerase solution 1x, 1.5 mM of Mg2+, 1 µl of each primer 10 µM, dideoxynucleotides (dNTPs) of 2.5 mM and bidistilled water up to 20 µl of total volume. PCR conditions were: a cycle at 95ºC for 5 min; 35 cycles at 95ºC for 30s, 55ºC 30s, 72ºC 30s, and a final cycle at 72ºC during 10 min. Purification and sequencing were made as explained above.

Analyses of sequences

The sequences obtained were edited using BioEdit program[20] and compared with GenBank database (www.ncbi.nlm.nih.gov/genbank/) using BLAST Nucleotide tool (nBLAST). Species assignment was done to the best match reference sequence within GenBank. Species assignation was confirmed from phylogenetic methodology. A Neighbor-Joining tree containing the problem sequences and reference sequences from GenBank was reconstructed with MEGA version 6,[21] with Tamura Nei model[22] and uniform substitution rates. Robustness of the Neighbor-Joining topology was assessed using 10,000 bootstrap replicates.

RESULTS

DNA yields obtained from the analyzed candies ranged between undetectable <0.0005ng/µl in seven products (samples #3, #7, #8, #10, #15, #17 and #18) to 0.328ng/µl in the Portuguese gelatin of sample#6 (Table 1). Positive PCR amplification of one or the two assayed markers occurred from all except one product, fish gummies

(sample#3, Table 1). This means that DNA was present at least in 17 out of 18 samples (94.4%), although in very low (undetectable) quantity in six of them.

In the cases of successful DNA amplification, clean negative controls were obtained in all PCR (e.g., Fig. 2). The number of sequences retrieved in total from the 17 candies with positive PCR amplification was 118 for the 16S rDNA gene and 94 for the rbcL gene. The sequences were submitted to the European Nucleotide Archive (ENA), from the European Bioinformatics Institute in the European Molecular Biology Laboratory, EMBL-EBI (www.ebi.ac.uk/). Their accession numbers are HG964177-HG964248. For the 16S rRNA gene, amplicons ranged 116-122 bp in length and exhibited nucleotide polymorphisms corresponding to 13 haplotypes that allowed to unambiguously identifying five animal species to species level: cow *Bos taurus*, pig *Sus scrofa*, chicken *Gallus gallus*, deep Cape hake *Merluccius paradoxus*, human *Homo sapiens*. One haplotype could be assigned only at genus level (hake *Merluccius* sp.). For the rbcL gene, 159 bp long amplicons were obtained representing 14 haplotypes. Seven species were unambiguously identified from the haplotypes found for this DNA region: maize *Zea mays*, soya *Glicine max*, cacao *Theobroma cacao*, onion *Allium cepa*, tobacco *Nicotiana tabacum* and chestnut *Castanea sativa.* One haplotype exhibited the same E-value for two match hits with references sequences of wheat (*Triticum* sp.) and rye (*Secale cereale*), of the family Poaceae, and another with cumin (*Cuminum cyminum*) and carrot (*Daucus carota*), of the family Apiaceae. The remaining haplotypes could taxonomically assign the sequences only at genus (beans, *Vicia* sp.; rice, *Oryza* sp.; honeybush, *Ciclopia* sp.) or family level (Oleaceae). In spite of the short length of the two sequences here employed, the reconstructed Neighbor-Joining trees exhibited a rather good phylogenetic signal, grouping together the haplotypes of the same species or genus in the tree reconstructed from 16S rDNA sequences (Fig. 3). Likewise, the tree

reconstructed from rbcL sequences clustered haplotypes by family (Fig. 4); however, and as expected from their shorter length, bootstrapping values were lower than in the 16S rDNA-based tree, and some phylogenetic discrepancies occurred; for example, the sequences identified as *A. cepa* (onion), expected to be clustered with other Liliopsida such as the cereals, were alone in a branch apart.

Regarding candies composition, from 16S rDNA one animal species was found for most samples (Table 2), pig (*S. scrofa*) being the most frequent (in 64.7% of candies) followed by cow (*B. taurus*) (47.1% of candies), hake (genus *Merluccius*) (11.8%) and chicken (*G. gallus*) (5.5%). From rbcL sequences (Table 2) a mixture of at least three plant species was detected in eight of the samples analyzed (44.4%). Seven samples contained maize (*Z. mays*) and also seven samples contained beans or related species (*Vicia* sp.). Less frequent ingredients were cereals of the tribe of wheat/rye; soya, rice, honeybush and chestnut; tobacco, cacao, cumin and Oleaceae (found from five; two; and one samples respectively). Some ingredients were unexpected, since contamination from the analysis process can be discarded given clean negative PCR controls and strict measures of sterility, such as human DNA and tobacco found in eight (44% samples: #2, #7, #8, #11, #13, #14, #15 and #17) and one (#13) candies respectively (Table 2).

Many species detected from DNA were not stated in the labels and vice versa (Table 2). Only one of the samples analyzed provided DNA results concordant with the label: the gelatin powder of Sample#1 (Tables 1 and 2) that declared to contain porcine gelatin and contained porcine DNA. Other DNA-label concordances occurred in a few samples: #11 label also declared porcine gelatin and porcine DNA was found; #2 and #7 declared corn and contained corn DNA, as did occur in #8 for soya and #15 for wheat. In general there was a difference between the means of animal and plant species found only from DNA and only from labels (Fig. 5, Table 2). More animal species were found from

DNA than from labels with Horreo *et al*.[18] primers, whereas for plants it was the opposite, with more plants and fruits stated in the labels than found from DNA with Han *et al.* [19] primers.

Some cases found in this study can be problematic for consumers. Pig, not accepted by some religions, is one example. Pig traces appeared but were not declared in 55.6% of samples (Fig. 4). Moreover, two samples labeled as apt for vegetarians (vanilla custard, Sample#2; agar, Sample#5) contained pig traces. Another possible problematic case was the failure to declare fish content, although hake traces were present in three samples.

DISCUSSION

Our results revealed that DNA traces were present in most analyzed commercial candies, and that its quality and concentration was sufficient for successful PCR amplification of short DNA sequences of species-specific value. The results obtained here confirm the power of DNA tests for detecting traces of ingredients in complex food matrices, supporting other authors who used DNA for identifying unwanted species in candy, for instance Demirhan *et al*.[7] Most studies use specific markers for identifying only targeted species or DNA sequences; for example the mentioned study targeting porcine,[7] markers for detecting genetically modified maize and soy,[12] or saffron in highly processed products.[15] Here we have followed a different approach of PCR-cloning of conserved DNA sequences using universal primers, instead of species-targeted ones, because our objective was to detect as many species as possible. Indeed, not finding a species in only a dozen sequences (from cloning) cannot ensure that such species is absent from the product; it could be present in low proportion and remain undetected. Due to the many ingredients contained in candy, this process could be

considerably improved using next generation sequencing technology (NGST): high-throughput sequencing approaches after direct DNA extraction from a matrix or environmental sample. Capable to generate millions of sequences at the same time, NGST is now used in ecology for biodiversity monitoring, and its application in food sciences has been suggested for microbes in complex food matrices. [23,24]

It should to be taken into account that the absence of DNA traces of a species in a product does not imply that species is really absent; DNA can be so degraded that PCR may fail, and/or primers may fail to anneal if they are insufficiently specific for a taxonomic group. Conversely, if DNA traces of a species occur in a product and contamination can be reasonably discarded, as it is the present case, there is no doubt that the species is really present in that product. Despite quite limited sample size, in our results we found a surprising and unexpected high level of failure to declare species contents in commercial packed candies. Many undeclared species were detected in more than 90% of the analyzed candies, and some of them could raise ethical issues (pig; animal species in vegetarian candies) for many consumers. Since the candy trade is widely globalized, [25] and our study was done from candies made in five different countries, the results here obtained could likely be generalized.

Regulations of candy labelling are not homogeneous worldwide and each country has specific laws, generally focused on allergenic ingredients. For example, in the US it is mandatory to list major allergens contained in the ingredients on the label of packed food, including candy, stating the species the major allergen is derived from (Food Allergen Labelling and Consumer Protection Act of 2004; Public Law 108-282, Title II). In European common law, packed food must also display a list of potential allergens separated from the list of ingredients (EU 1169/2011). In addition to this general normative, at national level some regulations are specifically applied to candy; for

example, in Spain the Royal Decree 1245/2008 states that packed candies must exhibit information about allergens on the label. The results of the present study suggest that the current labeling normative should be improved. At least the species used for the gelatin should be disclosed. The consumer should be informed about fish, which can trigger allergic reactions if inadvertently eaten with candy. [11] On the other hand, pig and cow are frequently employed to produce commercial gelatin, [6,7] thus their presence in most candies here analyzed is not surprising. However, their occurrence in candies labelled "For vegetarians" could be considered a fraud and undermine the choice rights of vegetarian consumers.

The causes of failure for declaring all the species detected in this study are probably diverse. Some ingredients could have been merely listed as "colorants" or "spice", without disclosing the species contained. [26] In some cases it could be likely deliberate, as in Sample#5 supposedly made only from algae (colorants were not stated) but containing really pig, bean and honeybush traces. However in other cases the presence of some traces could be inadvertent. Some ingredients could have been accidentally acquired during the process of packing. [27] This could happen also in the cases of contamination with human DNA and even tobacco; it is very difficult to imagine that negligent manipulation of food products is deliberate. However, although likely not deliberate in some (perhaps in many) cases, the results found our study strongly support the need of a more careful control of the international candy market.

CONCLUSIONS

Using DNA analysis we have detected a generalized failure to inform about ingredients in commercial candies from five producer countries. DNA traces of many species undeclared in the labels like porcine, fish, soya, honeybush and others were found from

most analyzed samples. Undeclared porcine DNA was found in samples labeled as "For vegetarians", undermining the rights of vegetarian consumers. A more strict control of commercial candies is recommended, applying methodology based on DNA-tests or Next Generation Sequencing Technology, which could obtain higher resolution on the composition of these sweets.

ACKNOWLEDGEMENTS

This research was financially supported by Fundacion Mapfre (SV13-MAPFRE-1), Regional Government of the Principado de Asturias, Spain (Research Grant GRUPIN14-093) and Campus of Excellence of the University of Oviedo. M.M-C. holds a Spanish National Grant (reference AP-2010-5211).The data reported in this paper are archived at the following databases: European Nucleotide Archive (ENA), from the European Bioinformatics Institute in the European Molecular Biology Laboratory, EMBL-EBI (http://www.ebi.ac.uk/). We also acknowledge the assistance of Daniel Serna Fuente (Sequencing Unit of the University of Oviedo).

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TABLES

Table 1: summary of candy samples analyzed.

							PCR amplification	
S	**Country**	**Type**	**Animal species**	**Plant species**	**Specific indications**	**DNA (ng/µl)**	**16S rDNA**	**rbcL**
1	Spain/Portugal	Unflavored gelatin	Pork	No	ND	0.0384	+	-
2	France	Vanilla custard	ND	Maize	Vegetarian	0.172	+	+
3	Spain	Fish-shaped gummies	Possible milk, egg traces	Possible peanuts, tree nuts,wheat, soy traces	ND	<0.0005	-	-
4	France	Lemon gelatin dessert	Gelatin. Possible milk & egg traces	Possible soy, nuts traces	Possible gluten content	0.0124	+	+
5	Spain/Portugal	Vegetable gelatin	ND	Agar-agar	Vegan, Agar 100%	0.0258	+	+
6	Portugal	Neutral gelatin	Gelatin	ND	ND	0.328	-	+
7	Spain	Marshmallows	Gelatin	Corn; Arabic gum	Gluten-free	<0.0005	+	+

8	Spain	Strawberry bubble gum	ND	Soya, arabic gum	ND	<0.0005	+	+
9	Spain	Strawberry candy	Gelatin, cochineal	Cherry, lemon, pineapple, strawberry, orange, apple, blackcurrant, elder berry, aronia, grape, mango, passion fruit, carob, turmeric	ND	0.0238	+	-
10	Portugal	Watermelon candy	Gelatin	ND	ND	<0.0005	+	+
11	Portugal	Marshmallows	Porcine gelatin	Wheat, corn	ND	0.0226	+	+
12	Spain	Green soft jelly	ND	Lemon, orange, strawberry, apple, pineapple, safflower, potato, carrot, radish, hibiscus, blackcurrant, spirulina.	Gluten free	0.0484	+	+
13	Spain	Color gummies	Gelatin	Carrot, blackcurrant, paprika, turmeric	ND	0.0164	+	+
14	Sweden	Gummies	Gelatin	Licorice, peanuts	ND	0.0174	+	+

15	Portugal	Pineapple gelatin dessert	Gelatin; Possible eggs & milk traces	Possible wheat traces	ND	<0.0005	+	+
16	Spain	Strawberry candy	Gelatin, cochineal	Cherry, lemon, pineapple, strawberry, orange, apple, blackcurrant, elder berry, aronia, grape, mango, passion fruit, carob, turmeric	ND	0.0208	+	+
17	Spain	Sherbet powder	ND	ND	Sugar & flavours	<0.0005	+	-
18	Turkey/Spain	Fruit bubble gum	Gelatin	Watermelon, pineapple, melon, arabic gum, turmeric	Contains Brilliant Blue FCF	<0.0005	+	+

Country of origin, type of product, animal and plant species declared in the label, specific indications for consumers, DNA quantity determined by fluorometry, PCR amplification of the markers assayed (positive or negative as visualized in agarose gel).

Table 2: Composition of the analyzed candy samples as identified from DNA.

	Nº species																	
Sample	Animals	Plants	Clones	Cow	Pork	Hake	Chicken	Beans	Rice	Cereals	Apiaceae	Oleaceae	Onion	Chestnut	Honeybush	Soya	Cacao	Corn	Contaminants
1	1 (1)	0 (0)	8	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2 (veg)	1 (0)	2 (1)	11	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	+
3	0 (2)	0 (4)	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	2 (3)	4 (2)	14	+	+	-	-	+	-	+	-	-	+	-	-	-	+	-	-
5 (veg)	1 (0)	2 (1)	12	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-
6	0 (1)	3 (0)	6	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-
7	4 (1)	1 (2)	16	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+
8	3 (0)	2 (2)	10	+	+	+	-	+	-	-	-	-	-	-	-	+	-	-	+
9	1 (2)	0 (14)	5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

10	1 (1)	3 (0)	13	+	-	-	-	+	-	+	-	-	-	-	-	-	-	+	-
11	1 (1)	0 (2)	8	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
12	1 (0)	3 (12)	17	+	-	-	-	+	-	-	-	+	-	-	+	-	-	-	-
13	2 (1)	2 (4)	14	+	+	-	-	+	-	-	-	-	-	-	-	-	-	+	+
14	2 (1)	3 (2)	15	+	+	-	-	-	-	+	-	-	-	-	-	+	-	+	+
15	2 (3)	2 (1)	17	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	+
16	1 (2)	3 (14)	19	+	-	-	-	-	-	+	-	-	-	+	-	-	-	+	-
17	1 (0)	0 (0)	4	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
18	1 (1)	3 (5)	23	-	+	-	-	-	-	-	-	-	-	+	+	-	-	+	-

Number of animal and plant species from DNA (declared in the label in parenthesis); number of clone sequences obtained from each sample; presence/absence of different ingredients authenticated from DNA sequences as +/-. Samples indicated for vegetarians are marked with (veg)

LEGENDS OF FIGURES

Figure 1: Photographs of different samples of this study: (1) Sample#1, gelatin powder (2) Sample#3, fish gummy; (3) Sample#2, pre-cooked mix for vanilla custard (4); Sample#4, lemon jelly; (5) Sample#8, strawberry gum; (6) Sample#7, marshmallow; (7) Sample#9, strawberry candy; (8) Sample#10, watermelon jelly; (9) Sample#12, green candy; (10) Sample#18, fruit gum; (11) Sample#11, marshmallow; (12) Sample#14, candy; (13) Sample#13, soft candy.

Figure 2: Photography of 2% agarose gel stained with ethidium bromide and visualized under UV light showing PCR products obtained with the 16S-H (A) and Rbc-L (B) primers; from left to right in A gel: positive control, samples 8-11 (two lanes/sample), negative control, empty lane, DNA ladder (marker of size, in base pairs); B gel: samples 8-11 (two lanes/sample), positive control, DNA ladder and negative control.

Figure 3: Neighbor-Joining tree reconstructed from the 16S rDNA sequences obtained from the analyzed candy. The haplotype name is followed by the closest taxonomic match. Reference sequences are included indicated as ref_ with their GenBank accession number. A sequence of the limpet *Patella depressa* was employed as outgroup.

Figure 4. Neighbor-Joining tree reconstructed from the rbcL sequences obtained from the analyzed candy. The haplotype name is followed by the closest taxonomic match. Reference sequences are included indicated as ref_ with their GenBank accession number. A sequence of the green alga *Fucus vesiculosus* was employed as outgroup.

Figure 5: Mean number per candy sample of animal and plant species of the following types: concordant, declared only in the labels and found only from DNA.

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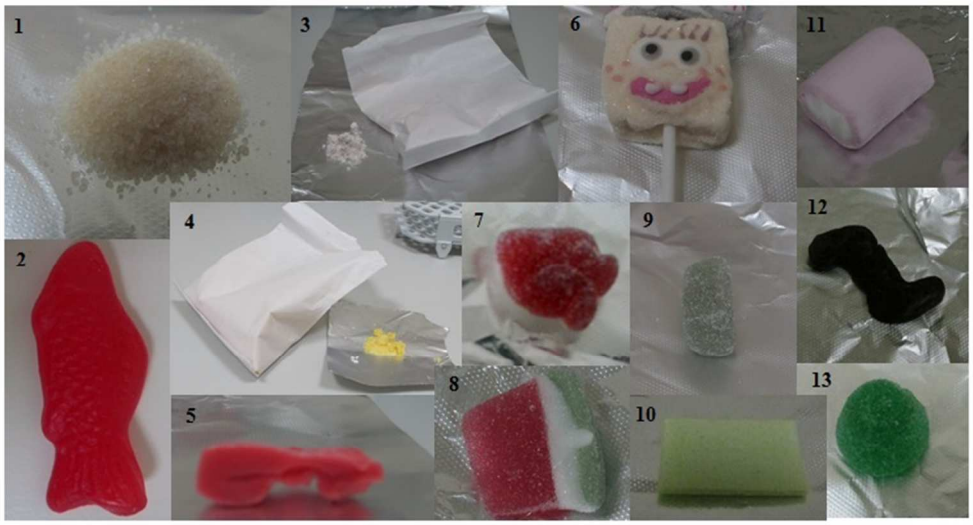

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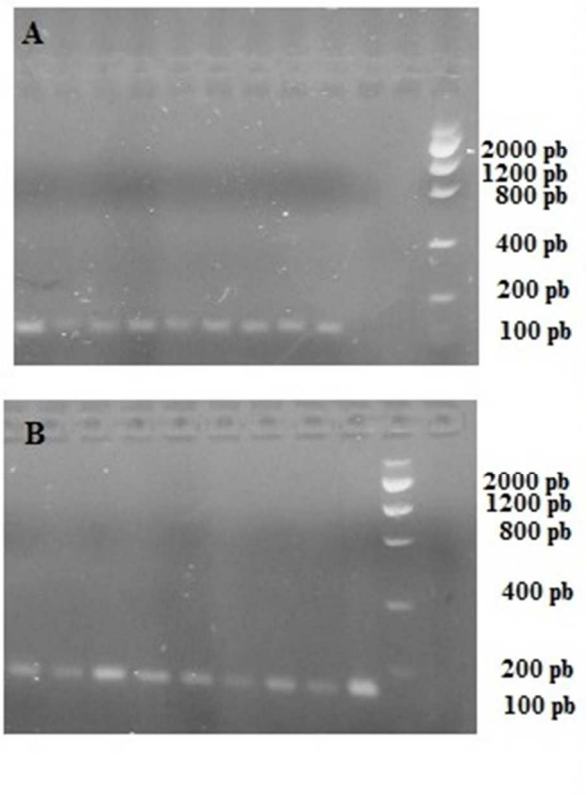

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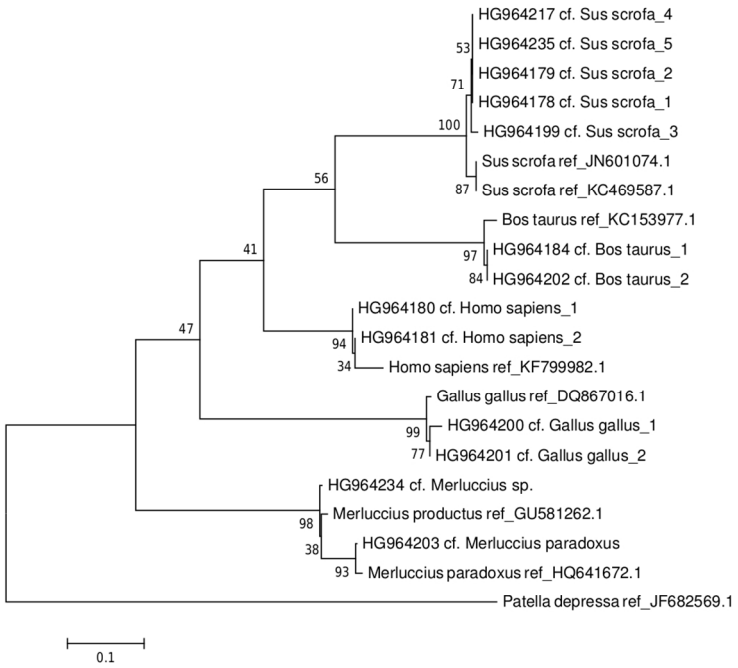

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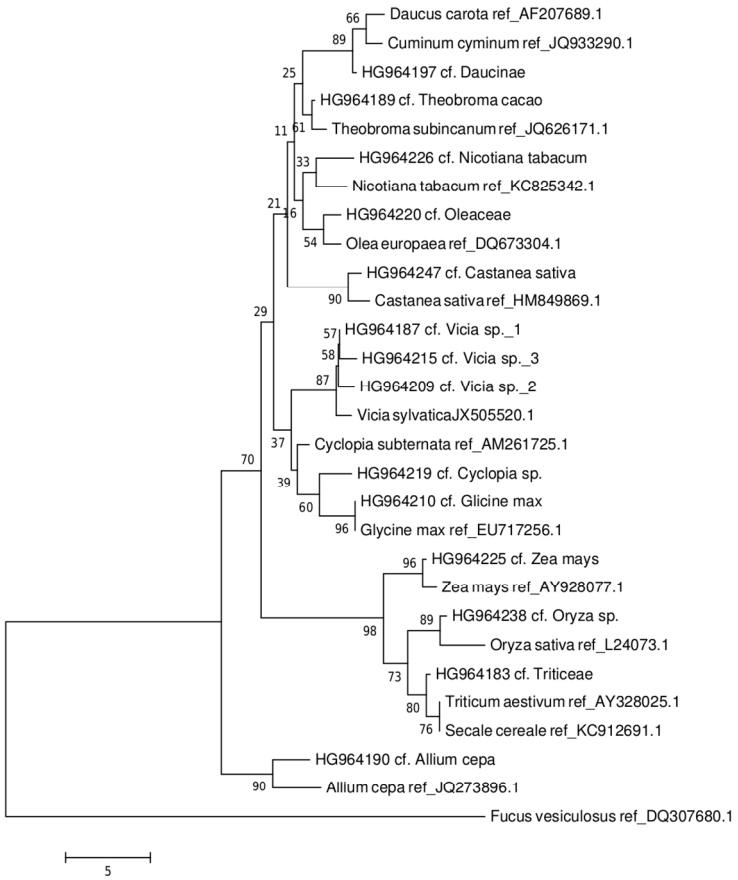

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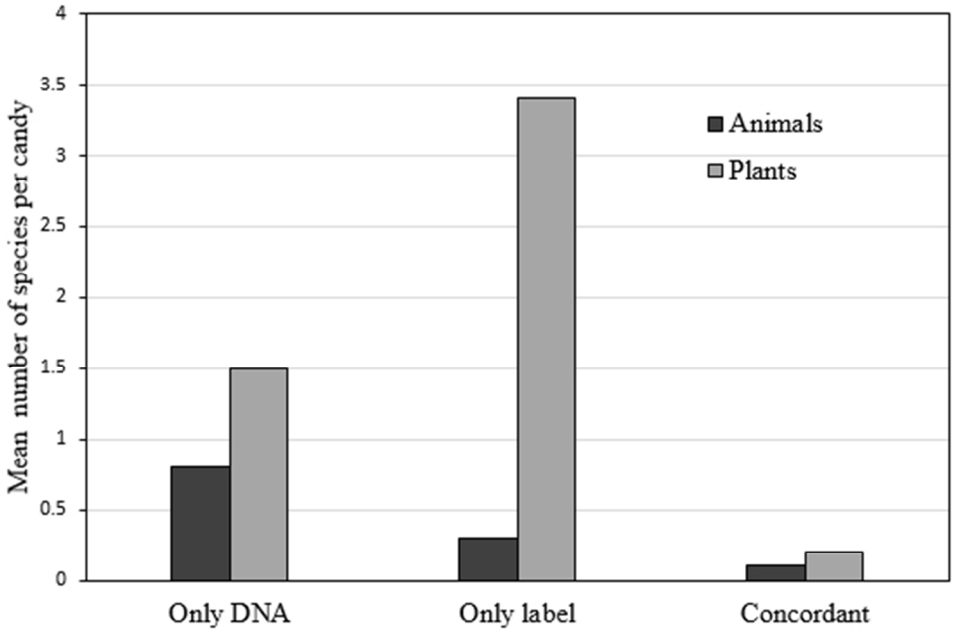

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