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Research paper

Species identification in forensic samples using the SPInDel approach: A GHEP-ISFG inter-laboratory collaborative exercise

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ABSTRACT

DNA is a powerful tool available for forensic investigations requiring identification of species. However, it is necessary to develop and validate methods able to produce results in degraded and or low quality DNA samples with the high standards obligatory in forensic research. Here, we describe a voluntary collaborative exercise to test the recently developed Species Identification by Insertions/Deletions

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(SPInDel) method. The SPInDel kit allows the identification of species by the generation of numeric profiles combining the lengths of six mitochondrial ribosomal RNA (rRNA) gene regions amplified in a single reaction followed by capillary electrophoresis. The exercise was organized during 2014 by a Working Commission of the Spanish and Portuguese-Speaking Working Group of the International Society for Forensic Genetics (GHEP-ISFG), created in 2013. The 24 participating laboratories from 10 countries were asked to identify the species in 11 DNA samples from previous GHEP-ISFG proficiency tests using a SPInDel primer mix and control samples of the 10 target species. A computer software was also provided to the participants to assist the analyses of the results. All samples were correctly identified by 22 of the 24 laboratories, including samples with low amounts of DNA (hair shafts) and mixtures of saliva and blood. Correct species identifications were obtained in 238 of the 241 (98.8%) reported SPInDel profiles. Two laboratories were responsible for the three cases of misclassifications. The SPInDel was efficient in the identification of species in mixtures considering that only a single laboratory failed to detect a mixture in one sample. This result suggests that SPInDel is a valid method for mixture analyses without the need for DNA sequencing, with the advantage of identifying more than one species in a single reaction. The low frequency of wrong (5.0%) and missing (2.1%) alleles did not interfere with the correct species identification, which demonstrated the advantage of using a method based on the analysis of multiple loci. Overall, the SPInDel method was easily implemented by laboratories using different genotyping platforms, the interpretation of results was straightforward and the SPInDel software was used without any problems. The results of this collaborative exercise indicate that the SPInDel method can be applied successfully in forensic casework investigations.

1. Introduction

There is an increasing relevance of non-human DNA typing in forensic science investigations. In particular, the identification of species is an area of growing relevance in the investigation of wildlife poaching, illegal trade of protected species, fraudulent labeling of food products, trace materials left on crime scenes, among other situations [1–5]. The laboratory procedures used in such cases often comprise the extraction of DNA from the casework sample, the PCR amplification using species-specific or universal primers and DNA sequencing [6–9]. However, the implementation of Sanger or next-generation DNA sequencing in routine casework samples is expensive to many laboratories and is unable to provide clear results in the presence of complex mixtures or in degraded samples [10,11].

The Species Identification by Insertions/Deletions (SPInDel) method is an alternative to DNA sequencing that uses a conventional genotyping methodology similar to that employed with Short Tandem Repeats (STRs), involving multiplex PCR followed by fragment size determination using capillary electrophoresis [12]. The identification of species with the SPInDel method is achieved by the amplification of six hypervariable regions in mitochondrial ribosomal RNA (rRNA) genes using highly conserved PCR primers (Fig. 1). Each species is defined by a unique numeric profile of fragment lengths (i.e., a numeric barcode) resulting from the combination of the length of indel-rich regions (Fig. 2, Table 1). The SPInDel multiplex PCR was developed for identification of humans and the most common domestic animal species [12], although it works well on other species [13]. A SPInDel computational workbench was also built to help researchers in all steps of the species identification process [14].

The potential utility of the SPInDel methodology for species identification in forensic casework samples prompted us to carry out an inter-laboratory collaborative exercise within a Working Commission of the Spanish and Portuguese-Speaking Working Group of the International Society for Forensic Genetics (GHEP-ISFG) created solely for this purpose. This Commission was proposed and approved at the General Assembly during the XVIII GHEP-ISFG Annual Meeting held in Seville (Spain) in September 2013 and the preliminary results were presented at the 26th ISFG World Congress in September 2015 [15]. The aims of this voluntary collaborative exercise were to test the reliability of the SPInDel kit on low-quality DNA samples and assess its accuracy when used by different laboratories. The exercise also evaluated the usefulness of the SPInDel computational workbench for data analyses.

2. Materials and methods

The SPInDel collaborative exercise was opened to all laboratories with GHEP-ISFG members. Twenty-four laboratories from 10 countries participated in the collaborative exercise (Supplementary Table S1). The laboratories were asked to analyze the eight samples (identified as M1 to M8) included in the 2014 GHEP-ISFG proficiency test (http://ghep-isfg.org/en/proficiency/) and three samples with non-human material from previous GHEP-ISFG proficiency tests (samples M7 from 2011, M7 from 2012 and M8 from 2013), as shown in Table 2. The samples were from human (*Homo sapiens*), cat (*Felis catus*), dog (*Canis familiaris*) and mixtures of human and cow (*Bos taurus*) and human and horse (*Equus caballus*). Some of the samples had low quantity and/or quality DNA, as often encountered in forensic investigations. For instance, sample M4 (2014) was a mixture 2:1 (v/v) of saliva and blood from a human male and a human female respectively, on a paper napkin; sample M5 (2014) was body hair from a human male donor; and sample M7 (2014) was a mixture 4:1 (v/v) of blood from a wild cow and saliva from a human male donor on a nylon flocked swab.

The participating laboratories were supplied with a SPInDel primer mix for the multiplex PCR amplification of six

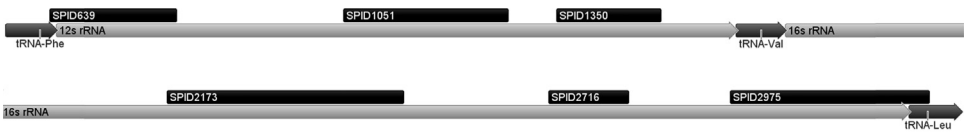

Fig. 1. Graphical representation of the six mitochondrial ribosomal RNA (rRNA) gene regions (black bars) amplified by the SPInDel multiplex PCR.

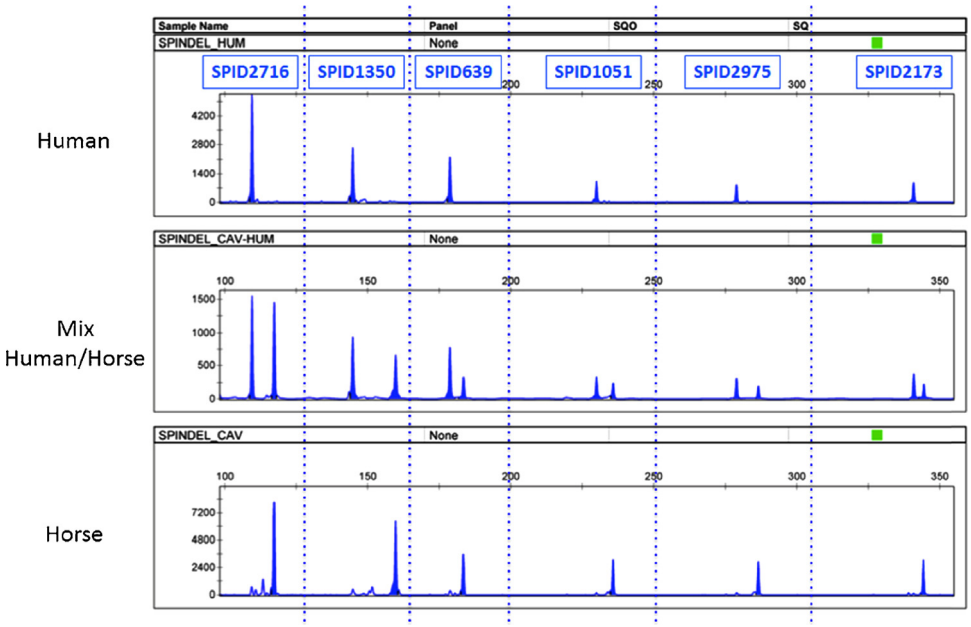

Fig. 2. Examples of electropherograms obtained with the SPInDel multiplex PCR.

Table 1
List of species targeted by the SPInDel multiplex PCR. The reference SPInDel profiles are indicated for each species.

Species	Markers					
	SPID2716	SPID1350	SPID639	SPID1051	SPID2975	SPID2173
Capra hircus (Goat)	11	15	12	14	13	14
Canis lupus familiaris (Dog)	19	11	12	13	19	15
Equus caballus (Horse)	18	25	16	15	17	15
Oryctolagus cuniculus (Rabbit)	17	11	12	13	16	17
Felis catus (Cat)	13	13	14	15	19	12
Homo sapiens (Human)	10	10	11	10	10	11
Ovis aries (Sheep)	11	15	13	14	12	16
Sus scrofa (Pig)	14	17	13	13	16	10
Mus musculus (Mouse)	12	10	11	15	15	14
Bos taurus (Cattle)	11	16	10	14	11	14

Table 2
List of forensic samples used in the collaborative exercise. The reference SPInDel profiles are indicated.

Sample	Species	SPInDel profile
M1–M6, M8 (2014)	*Homo sapiens*	(10, 10, 11, 10, 10, 11)
M7 (2014)	*Homo sapiens*	(10, 10, 11, 10, 10, 11)
	Bos taurus (mixture)	(11, 16, 10, 14, 11, 14)
M7 (2011)	*Canis lupus familiaris*	(19, 11, 12, 13, 19, 15)
M7 (2012)	*Felis catus*	(13, 13, 14, 15, 19, 12)
M8 (2013)	*Homo sapiens*	(10, 10, 11, 10, 10, 11)
	Equus caballus (mixture)	(18, 25, 16, 15, 17, 15)

hypervariable rRNA gene regions (Fig. 1; Supplementary Table S2). The original multiplex PCR described by Pereira *et al.* in 2010 [12] was modified by the removal of the two longest markers ('FG' and 'AC') and the addition of a shorter marker (SPID639) to increase the genotyping success in samples with degraded DNA. All markers are now fluorescently labeled with the 6-Carboxyfluorescein (6-FAM) dye (Fig. 2). The name of each target region was also changed to include the 5' position of the forward primer according to the numbering of the human mitochondrial DNA (mtDNA) reference sequence (revised Cambridge Reference Sequence, rCRS; NC_012920.1).

The multiplex was designed for ten mammalian species (Table 1), which have unique numeric combinations of sequence lengths for the six target regions (a 'SPInDel profile'). A reference sample for each target species and the respective panel and bins for GeneMapper automatic analysis were provided to the laboratories to be used for allele assignments. The laboratory work comprised the PCR amplification of the six markers in a single reaction using the provided primer mix, optimized to obtain balanced reaction products. The laboratories were asked to use the PCR conditions previously described [12,13] and all the instructions were made available through the GHEP-ISFG website (https://ghep-isfg.org/en/working-commissions/spindels-indels-for-species-identification/). The amplified products should be analyzed by automated capillary electrophoresis, being recommended the use of a capillary sequencer such as an "ABI Genetic Analyzer" (310, 3100, 3130, 3500, 3730 or equivalent variants). The original protocol uses POP-7 as the separation polymer, although POP-6 or POP-4 were also allowed, always taking into account the consequent changes in the electrophoretic mobility (Supplementary Table S3).

The identification of the species was facilitated by the use of the SPInDel Workbench version 1.3 portable (http://www.portugene.com/SPInDel/SPInDel_web.html) [14].

3. Results and discussion

The annual GHEP-ISFG proficiency test is divided into two levels: basic and advanced levels. Each of these levels comprises two modules: kinship and forensic. The laboratories may choose to participate in both levels or just in the basic one, and in this last case they may opt to participate only in the kinship module. Therefore, considering the 2014 GHEP-ISFG proficiency test as reference, all the laboratories had access to the samples corresponding to the basic level/kinship module (samples M1 to M3), some participated in the basic level/both modules (samples M1 to M5) and only a few participated in the both levels/both modules (samples M1 to M8). The number of laboratories providing results for each sample varied from 14 for sample M8 (2013) to 24 for samples M1, M2 and M3 (Table 3). In most cases, the absence of data for some samples was due to their unavailability in the laboratory, as explained above. In other cases, there were laboratories that did not report the SPInDel profile for a specific sample although it was available. For example, the same number of results for samples M4 and M5 were to be expected since all laboratories participating in the basic level/both modules have access to both samples. However, 20 laboratories reported results for M4 while 16 participants reported results for M5. In this case, since M5 was a hair sample (only for mtDNA typing) it is likely that the laboratories not routinely performing mtDNA analysis did not attempt to genotype it.

All laboratories presented the results in the correct format by using the SPInDel workbench. The instructions provided by the organizers of the exercise for using the software were sufficient and no further explanations or corrections were necessary. The main results are shown in Table 3. The global scenario is that 22 of the 24 laboratories reported correct species identifications for the 13 expected profiles (11 samples). Two laboratories were responsible for the three misidentifications (in samples M4, M5 and M8). Overall, concordance was obtained in 98.8% of the total reported results. For each profile, the level of concordance in identifications was always higher than 93%, including in samples with low amounts of DNA (hair shafts) and mixtures of saliva and blood. With the exception of a single laboratory, all laboratories correctly identified the species present in the mixtures. For instance, the 19 laboratories analyzing the sample M7 (2014) correctly identified the presence of DNA from both *H. sapiens* and *B. taurus*.

Out of the 241 reported profiles, 238 (98.8%) yielded correct species identification. The three cases of misclassifications represented 1.2% of the total. Two of these cases were the erroneous *C. hircus* detection in a *H. sapiens* sample. These errors occurred in the same laboratory and a possible explanation could be a contamination from the *C. hircus* control reference sample. This laboratory received the control sample tubes opened and dried out due to problems in the mail transportation and/or customs handling which may very likely have caused cross-contamination between samples. Furthermore, the fact that *C. hircus* and *H. sapiens* profiles diverge in all markers suggests that a contamination in control samples is more likely than an error in data analysis. The other case of misclassification was due to the absence of detection of *E. caballus* in a mixture. This missing profile occurred in a sample (M8 of 2013) with DNA extracted from a blood mixture of *E. caballus* and *H. sapiens* delivered on a piece of cleaning cloth. The low amounts of DNA in this sample might have led to problems in the interpretation of the profile or the use of POP4 as a separation polymer, which is a less viscous and lower-resolution polymer than POP-6 and POP-7 [16]. This laboratory and others using POP-4 described some problems associating the alleles from control samples into the corresponding bins provided in the exercise (Table 1; Supplementary Table S3), which might have caused some erroneous identifications. Nevertheless, the remaining 13 laboratories that analyzed sample M8 (2013) provided correct identification of the mixture.

The proportion of complete profiles with all correct alleles varied from 81.3% in sample M7 (2012) to 100% in samples M6 and M8 (2014) and M8 (2013), as shown in Table 3. Among 241 reported profiles, 12 had wrong alleles (5.0% of the total) and five had missing alleles (2.1% of the total). The highest number of wrong or missing alleles (3 cases each) was observed in samples M1 (2014), M2 (2014) and M7 (2012). The frequency of wrong (5.0%) and missing (2.1%) alleles was low and did not interfere with the correct species identification, mainly because the SPInDel method relies on the analysis of multiple loci [12], a clear advantage over methods targeting a single locus.

When considering the number of profiles with wrong alleles *per* marker, 16 out of 1446 reported alleles were wrong (1.1% of the total). The wrong alleles were reported in three markers: SPID2716, SPID639 and SPID2173 (Table 4). The marker SPID639 had the

Table 3
Main results of the collaborative exercise for species identification using the SPInDel multiplex PCR. The two profiles (1st pr. and 2nd pr.) of the species present in samples with mixtures (M7 of 2014 and M8 of 2013) are described separately.

Samples	Number of reported profiles	Correct species identifications	Complete profiles with all correct alleles	Profiles with wrong alleles	Profiles with missing alleles
M1 (2014)	24	24 (100%)	21 (87.5%)	3 (12.5%)	0
M2 (2014)	24	24 (100%)	21 (87.5%)	2 (8.3%)	1 (4.2%)
M3 (2014)	24	24 (100%)	23 (95.8%)	1 (4.2%)	0
M4 (2014)	20	19 (95.0%)	19 (95.0%)	1 (5.0%)	0
M5 (2014)	16	15 (93.8%)	15 (93.8%)	0	1 (6.3%)
M6 (2014)	19	19 (100%)	19 (100%)	0	0
M7 (2014) 1st pr.	19	19 (100%)	17 (89.5%)	2 (10.5%)	0
M7 (2014) 2nd pr.	19	19 (100%)	18 (94.7%)	1 (5.3%)	0
M8 (2014)	17	17 (100%)	17 (100%)	0	0
M7 (2011)	15	15 (100%)	14 (93.3%)	1 (6.7%)	0
M7 (2012)	16	16 (100%)	13 (81.3%)	1 (6.3%)	2 (12.5%)
M8 (2013) 1st pr.	14	14 (100%)	14 (100%)	0	0
M8 (2013) 2nd pr.	14	13 (92.9%)	13 (92.9%)	0	1 (7.1%)
Total	241	238 (98.8%)	224 (92.9%)	12 (5.0%)	5 (2.1%)

Table 4
Number of profiles with wrong (Wr.) and missing (Mi.) alleles *per* SPInDel marker reported by the 24 participating laboratories.

Markers	SPID2716		SPID1350		SPID639		SPID1051		SPID2975		SPID2173		Total	
Samples	Wr.	Mi.	Wr.	Mi.	Wr.	Mi.	Wr.	Mi.	Wr.	Mi.	Wr.	Mi.	Wr.	Mi.
M1 (2014)					2						2		4	0
M2 (2014)					2	1		1		1		1	2	4
M3 (2014)	1				1								2	0
M4 (2014)	1				1								2	0
M5 (2014)		1											0	1
M6 (2014)													0	0
M7 (2014) 1st pr.					1						2		3	0
M7 (2014) 2nd pr.					1								1	0
M8 (2014)													0	0
M7 (2011)											1		1	0
M7 (2012)		1									1	1	1	2
M8 (2013) 1st pr.													0	0
M8 (2013) 2nd pr.		1		1		1		1		1		1	0	6
Total	2	3	0	1	8	2	0	2	0	2	6	3	16	13

largest number of wrong alleles, with eight reported cases: six cases reported allele 12 instead of 11 for *H. sapiens*, one case reported allele 10 instead of 11 for *H. sapiens* and one case reported allele 11 instead of 10 for *B. taurus*. This problem is most probably due to allele binning misalignments. Since most of the alleles for each locus differ in size by 1 bp, slight variations in electrophoretic conditions may significantly alter migration. Moreover, it is well known that fragments with the same size differing in DNA sequence (as is the case of SPInDel mtDNA fragments) will show differences in electrophoretic mobility, therefore, it is very important to run control profiles as often as possible for correct profile determination of unknown samples.

In terms of profiles with missing alleles *per* marker, 13 out of 1446 reported alleles were missing (0.9% of the total). The four missing alleles for sample M2 (2014) were reported in a single profile (10, 10, 0, 0, 0, 0). Nevertheless, a correct identification (*H. sapiens*) was possible due to the redundant information provided by the SPInDel approach. There was no clear association between the frequency of missing alleles and the marker length (except for the case described above), with missing alleles detected in all markers.

Overall, only three SPInDel markers were reported with more than one error (wrong or missing allele): SPID2716, SPID639 and SPID2173 (Table 4). The marker with the largest number of errors was SPID639, with eight errors observed in different samples. The SPID639 error rate only represented 3.3% of allele determinations and seems to be mostly due to allele binning misalignments. This problem was worst when using lower-resolution polymers like POP-4, as reported by some laboratories.

The distribution of errors in profiles *per* laboratory, including profiles with wrong alleles ($n = 12$), missing alleles ($n = 5$) and incorrect identifications ($n = 3$), indicates that 14 laboratories (out of 24) provided results with no errors. Most reported errors were concentrated in four laboratories (each with 2 or more errors), while a single laboratory reported errors in all the six reported profiles. The few cases with wrong or missing alleles were reported by 10 laboratories, although yielding correct identifications in most cases due to the redundancy of the SPInDel method.

4. Conclusion

The collaborative exercises organized by the GHEP-ISFG since 1992 have proven to be important for the implementation and validation of new techniques and genetic markers, quality control and quality assurance, unification of criteria and as a proof of competence of the participating laboratories. The GHEP-ISFG exercises are also of general interest for the scientific community, as demonstrated by the number of resulting publications in peer-reviewed journals (e.g., [17–21]) and have played a pioneering role in the quality improvement of non-human DNA analyses. The present exercise was in line with the GHEP-ISFG collaborative strategy with a high level of participation and interest in the implementation of the SPInDel technique. The correct procedures in data analyses were followed by all users and all reports were accurate. The SPInDel software helped in data analysis and species identification, being used without any problem and no questions or doubts were raised to the organizers.

The high percentage of correct species identification obtained in complex DNA samples suggests that the SPInDel kit can be a valuable tool for forensic laboratories dealing with non-human DNA samples. The multiplex can serve as a preliminary test in samples suspected of being of non-human origin. The SPInDel kit can be particularly useful to discriminate among the ten target species described in Table 1. The SPInDel profiles that differ from those of the ten target species cannot be immediately identified, but such information can serve to guide further investigations in the laboratory, such as the use of specific PCR primers. Although the multiplex was originally designed for ten species, successful PCR amplifications are obtained in samples of diverse mammals [13] and even of birds and fishes [12]. Nevertheless, further studies are necessary to identify the SPInDel profiles of additional species. In any case, the SPInDel workbench includes hundreds of reference rRNA sequences that can provide the SPInDel profiles and help in the species identification.

The difficulties observed in the delivery of the reagents and samples to some countries may have caused the degradation and/or contamination of reference samples and primer mix, which may explain some of the wrong identifications. The single laboratory reporting errors in all profiles received the reference samples in poor and damaging conditions. Despite the obvious problems in sample transportation, all results were accepted and analyzed by the organizers of the exercise. Future exercises would benefit from a faster and more efficient shipping service and customs clearance.

Overall, the SPInDel method was easily implemented by laboratories using different genotyping platforms (Supplementary Table S3). The use of POP-4 was considered a problem by some laboratories when analysing the reference DNA samples and bins provided by the organizers. It is therefore recommended the use of high-resolution polymers to facilitate the analysis and avoid errors, although most identifications using POP-4 were correct. The method proved to be efficient in the identification of species in diverse forensic samples, including some with low amount and

degraded DNA. There was no relationship between the occurrence of missing alleles and the length of the marker, suggesting that the SPInDel markers with a length above 200 bp might also be useful even in degraded forensic samples. The use of mtDNA by the SPInDel approach also facilitates the analyses of low quality samples due to the high number of mtDNA molecules per cell. The success on the identification of species in mixtures demonstrate the advantage of using the SPInDel method when compared with conventional DNA sequencing, considering that the identification of both species is possible in a single reaction. The multilocus approach also proved to be very effective, providing correct identifications even with incomplete or partially correct results.

Finally, several participants demonstrated interest in using the SPInDel method regularly in their laboratories (some have declared to having already employed it in real cases), and all provided a positive feedback about the exercise.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2017.03.003.

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