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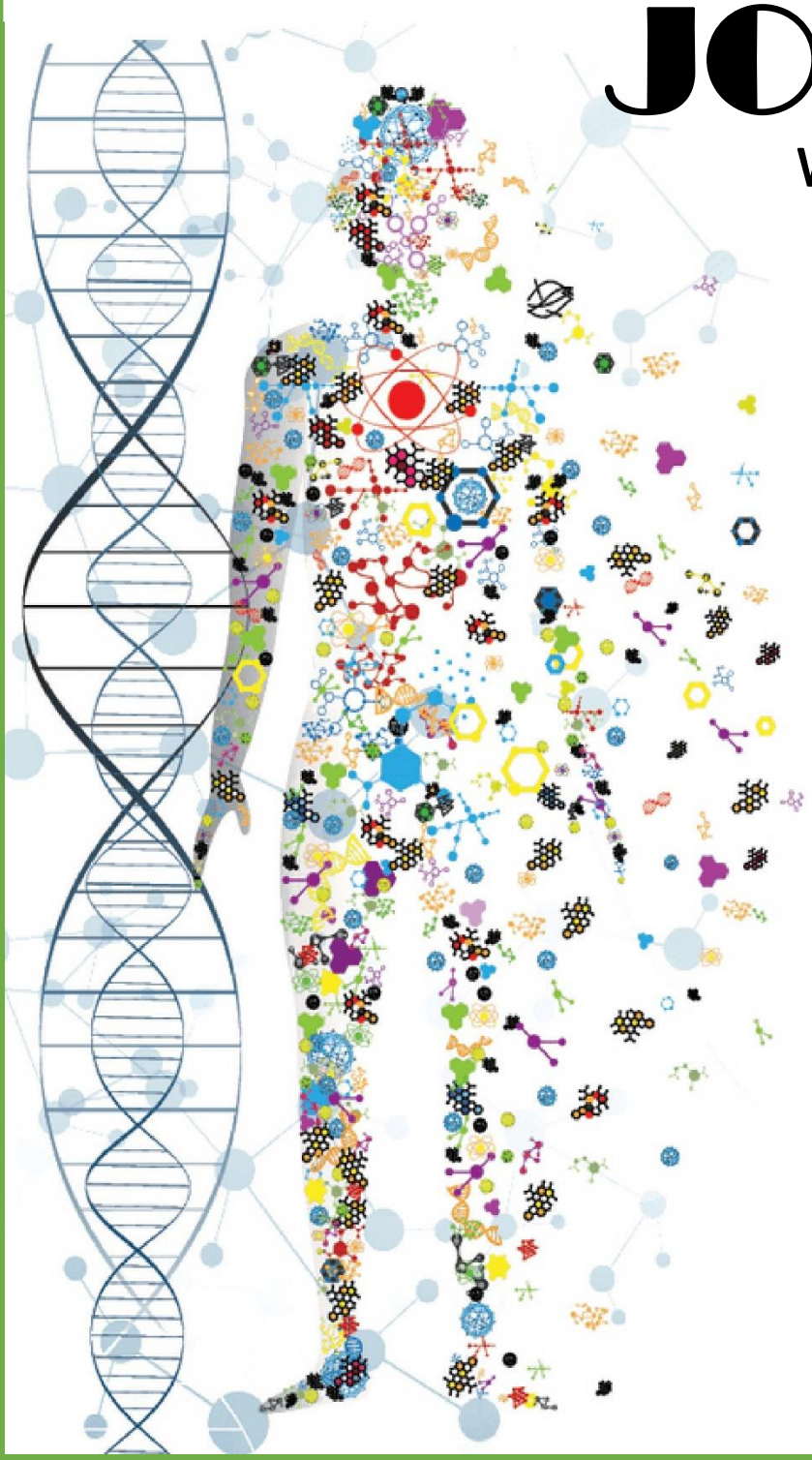

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MOLECULAR FORENSICS IN SILICO: DESIGNING LENGTH POLYMORPHISM BASED SPECIES IDENTIFIER FOLLOWED BY SPECIES-SPECIFIC PRIMERS FOR COW, TIGER, ELEPHANT AND RHINOCEROS

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ABSTRACT

Identification of species of biological samples is necessary in forensic investigations, particularly wildlife and illegal meat related crimes. Existing tools and techniques, includes PCR-RFLP, sequencing, and spectroscopic methods, an effective technique but often limited by cost, technical complexity, or turnaround time. In this in-silico study, mitochondrial DNA (mtDNA) from 21 mammalian species was analyzed to design a primer set (BV primer) capable of amplifying variable-length amplicon across various taxa provided preliminary species differentiation can be confirmed using restriction digestion pattern. To enhance resolution and forensic applicability, species-specific primers were further developed for Bos indicus, Panthera tigris, Elephas maximus, and Rhinoceros unicornis—species of high forensic relevance in India due to poaching or legal restrictions on cow slaughter. These primers possess amplification profiles unique for a species suitable for rapid and cost-effective identification. The approach reduces time, enhances reliability, and presents a scalable framework for developing additional species-specific primer sets, thereby strengthening forensic molecular tools for wildlife conservation and law enforcement.

INTRODUCTION:

In forensic laboratory, species identification of the biological samples is of utmost importance to move in investigation especially during a wildlife related crime. In India each state has its own law on cow slaughter. Majorly all north Indian states strictly bans the cow slaughter and its consumption (The Ban on Cow Slaughter Bill, 2019, 2019). Therefore, cow meat is often transported illegally claiming it to be of buffalo meat, making species identification a critical step. Mitochondrial DNA (Mt DNA) markers like cytochrome B (cyt B), Cytochrome c Oxidase subunit I (COI), 12s rRNA, 16s rRNA bas been used continuously in forensic labs for the species determination. In PCR RFLP, restriction digestion of 12s rRNA gene PCR product (using universal primers) giving different restriction pattern for different species[2,3,5]. Sequencing of cyt B gene is also widely used method for species identification[5,6]. Instrumental techniques have also been implied successfully, where detection is as easy as loading a questioned sample after proper sample preparation giving result within hours[7-12]. However, instrumental tools are not well established, require further optimization of protocol. It also requires properly trained personnel and cumbersome data interpretation. Kocher et al. (1989) introduced conserved primers for amplifying mitochondrial DNA genes including 12s rRNA across diverse vertebrate species[13].

Efficient amplification of DNA requires proper binding with the DNA. Ideally, a primer should have size of 18-25 bases with G/C content of 40-60% and 3' should end with G or C. The primer should show proper binding specially in last 5 bases from 3' end[14]. The melting temperature (Tm) should be 55-60 °C and annealing temperature (Ta) of 50-65 °C, 2-5 °C lower than Tm[15]. Self, homo and hetero-dimer should be non-significant (ΔG should be less negative; ideally between 0 to -6)[16]. Based on these criteria, 12s rRNA universal primer set was analyzed using integrated DNA technologies; OligoAnalyzer tool[17] summarized in Table 1.

These primers set often fails to undergo and efficient amplification of gene. To develop a more promising set of primer for species identification, surpassing all the criteria of a primer sets. The mitochondrial DNA size differs among species, here 21 mammalian species were considered for study. Table 2 contains the list of mammalian species with their mt DNA sizes.

METHODOLOGY:

The mt DNA of all 21 mammalian species were aligned using Clustal Omega [18]. Based on similarity in sequence one forward and reverse primer set (named "BV primer set") were designed which can amplify in all these mammalian species. The site of binding of these primers were identified using SnapGene (v.8.1) (http://www.snapgene.com/). The forward primer binds at 16,517th and reverse primer binds at 1,073rd in reference of human genome. It amplifies the sequence which shows higher size variability among species. Figure 1. Illustrates the binding and amplification of target location in mt DNA. Restriction digestion pattern of this amplicon is unique. Later, species-specific primer sets are developed for rhinoceros, cow, tiger and elephant (named as BVr, BVc, BVt, BVe respectively.

Name	5' to 3' sequence	Location (ref. to human mt DNA)	GC%	Tm in degree C	Highest ΔG for hairpin (in kcal/mol) with its Th	Highest ΔG for homo-dimer (in kcal/mol)	Highest ΔG for hetero-dimer (in kcal/mol)
Forward primer	CAA ACT GGG ATT AGA TAC CCC ACT AT	1066	42.3%	63.8	-2.16 Th= 47.5*	-8.09*	-7.48*
Reverse primer	GAG GGT GAC GGG CGG TGT GT	1478	70%*	70.3*	-0.24 Th=31.2	-3.61	-7.48*

Table 1. Fundamental analysis of this 12s rRNA universal primer

This the melting temperature of hairpin structure

***- Marks the failed parameters**

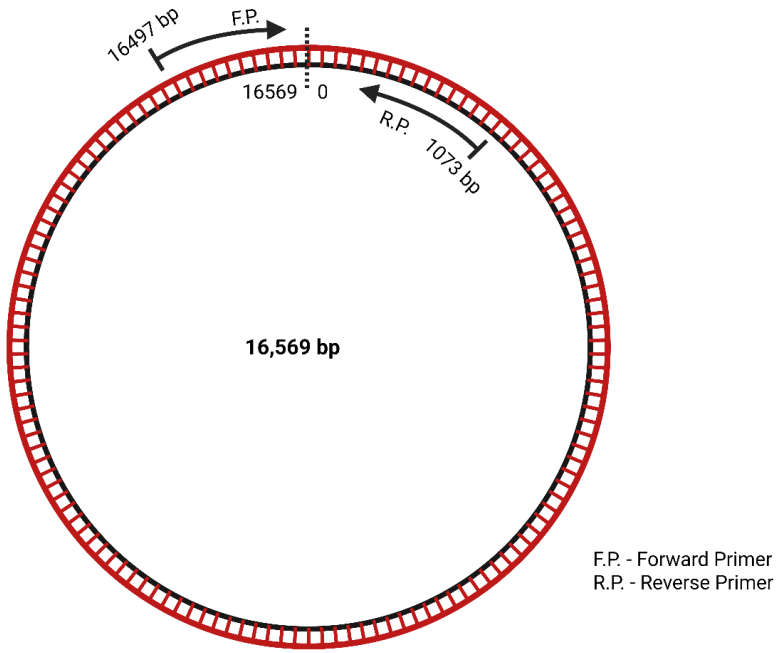

FIG-1. Human mitochondrial DNA showing the direction of amplification of BV primer set.

RESULTS:

The BV primer set were fundamentally analyzed using Integrated DNA Technologies, *OligoAnalyzer tool*[17]. Table 2 contains the result of fundamental analysis of BV primers.

Amplification direction	5' to 3' sequence	Size in bp	Location (Ref. of human sequence)	GC%	Tm in degree C	Highest Delta G for hairpin (in kcal/mol) with its Tm	Highest Delta G for homo-dimer (in kcal/mol)	Highest Delta G for hetero-dimer (in kcal/mol)
Forward	5'-CATCTGGTTC TTACTTCAGG-3'	20	16497	45%	57.5	-0.84 Th=34.2	-3.55	-3.07
Reverse	5'-GCATAGTGGG GTATCTAATCC-3'	21	1073	47.6%	59.3	0.42 Th= 18.6	-3.14	-3.07

Table 2. Fundamental analysis of BV primer set

The yielded amplicon size in each species is unique Table 3 shows the list of animal species with their amplicon sizes. Figure 2 shows 1% agarose gel simulation showing the species-specific amplicon position with respect to each other. However, the difference between the PCR product length is small making the species confirmation difficult. However, suspected species can be eliminated using human amplicon as reference. The difference in position of *Bos indicus* and *Canis lupus* can easily eliminate the possibilities of the questioned sample. Howbeit, similar is not true for *Bos indicus* and *Bubalus bubalis.* Polyacrylamide gel electrophoresis (PAGE) with higher resolving gel percentage can separate DNA fragment whose lengths differ by as little as 0.1% (i.e., 1 bp in 1000 bp)[19].

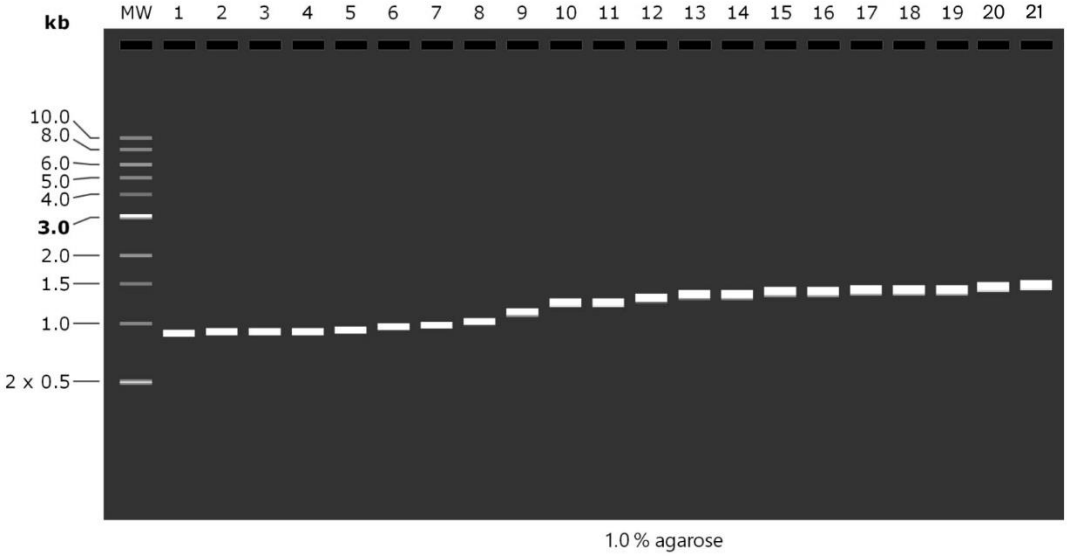

FIG-2. Simulated 1% agarose gel showing the amplicon placement in gel with respect to each other using SnapGene (v.8.1). (Numbers on top to be matched with table 3 for species name).

In similar amplicon sized species, restriction pattern makes the identification of species much easier. Table 3 also shows the restriction digestion sites of *BfaI, HhaI, ScaI, BshNI, AleI, ApoI* in PCR product. The restriction site in amplicon was identified using SnapGene (v.8.1). But restriction digestion alone has many disadvantages like star activity, incomplete digestion, sensitivity to methylations, sensitivity to temperature and pH[20,21]. Inability to detect smaller (<100bp) fragments in gel gives an added disadvantage.

Code	Species	Amplicon size (in bp)	BfaI	HhaI	ScaI	BshNI	AleI	ApoI
1.	*Bos indicus*	906	330, 460, 568 Size-330,130, 108, 338	NA	NA	NA	552 Size-552, 354	NA
2.	*Bubalus bubalis*	917	333, 470,579 Size- 333, 137, 109, 338					

3.	*Ovis aries*	921	335, 472 Size- 335, 137, 449	NA	NA	404 Size- 404, 517	NA	NA
4.	*Capra hircus*	927	343, 480 Size- 343, 137, 447	NA	NA	NA	NA	NA
5.	*Axis axis*	932	349, 487, 919 Size- 349, 138, 431, 14	223, 421 Size- 223, 198, 511	NA	NA	579 Size- 579, 353	NA
6.	*Balaenoptera musculus*	974	218, 369, 385,521, 670, 676 Size- 218, 151, 16, 136, 149, 6, 298	NA	265, 674 Size- 265, 409, 300	NA	772 Size- 772, 202	NA
7.	*Giraffa camelopardalis*	992	NA	636 Size- 636, 356	NA	NA	NA	NA
8.	*Bandicota indica*	1010	399, 732 Size- 399, 333, 278	NA	NA	NA	NA	NA
9.	*Homo sapiens*	1124	713, 845 Size- 713, 132, 279	NA	NA	NA	NA	NA
10.	*Panthera leo*	1228	773, 814, 1024, 1139 Size- 773, 41, 210, 115, 89	304, 306, 322 Size- 304, 2, 16, 906	629 Size- 629, 599	NA	NA	NA
11.	*Ursus thibetanus laniger*	1232	13, 772, 813 Size- 13, 759, 41, 419	861 Size- 861, 371	NA	NA	NA	NA
12.	*Equus ferus caballus*	1294	758, 840, 881, 992 Size- 758, 82, 41, 111, 302	505 Size- 505, 789	NA	NA	272, 932, 1042 Size- 272, 660, 110, 252	NA

13.	*Camelus dromedarius*	1334	772, 842, 897, 992 Size- 772, 342, 95, 70, 55	650, 874 Size- 650, 224, 460	NA	892 Size- 892, 442	1082 Size- 1082, 252	784, 798, 1143 Size- 784, 14, 345, 191
14.	*Susscrofa cristatus*	1351	750, 903, 1281, 1337 Size- 750, 153, 378, 56, 14	466, 992 Size- 466, 526, 359	NA	737 Size- 737, 614	1305 Size- 1305, 46	NA
15.	*Susscrofa domesticus*	1386	783, 936, 1316, 1372 Size- 783, 153, 380, 56, 14	562, 1025 Size- 562, 463, 361	NA	770 Size- 770, 616	1340 Size- 1340, 46	812 Size- 812, 574
16.	*Panthera pardus*	1394	895, 941, 982, 1305 Size- 895, 46, 41, 323, 89	303,305, 739 Size- 303, 2, 434, 655	NA	NA	NA	NA
17.	*Rhinoceros unicornis*	1405	823, 951, 992 Size- 823, 128, 41, 413	579, 585 Size- 579, 6, 820	NA	NA	1043, 1359	NA
18.	*Canis lupus*	1413	792, 1005 Size- 792, 213, 405	631 Size- 631, 782	NA	NA	1365	NA
19.	*Felis catus*	1416	650, 739,803, 962, 1327 Size- 650, 89, 64, 159, 365, 89	304, 306 Size- 304, 2, 110	NA	NA	NA	NA
20.	*Panthera tigris tigris*	1463	800, 1011, 1052, 1374 Size- 800, 211, 41, 322, 89	303, 305 Size- 303, 2, 1158	NA	NA	NA	NA
21.	*Panthera tigris*	1477	814, 1025, 1066, 1388 Size-	303, 305 Size- 303, 2, 1172	NA	NA	1225 Size- 1225, 252	NA

Table 3. Amplicon size of various species with BV primer and their restriction digestion pattern.

Species specific primer sets (see table 4) which shows amplification only with the mtDNA of that particular species. This is among the most reliable tool for species confirmation. Fundamental analysis of these primer showed no constraint. All animal specific primer can be used individually or as single cocktail. Primer sets specific to a species will give single band of size specific for that species. List of species-specific primer and its amplicon size is given in table 4.

Primer name	Target animal	Forward primer	Reverse primer	Amplicon Size
BVr	*Rhinoceros unicornis*	5'-AAAGCGTGTC AAAGATATAACC-3'	5'-GCATAGTGGG GTATCTAATCC-3'	153
BVc	*Bos indicus*	5'-GTGAGAATGC CCTCTAGGTTG-3'	5'-GCATAGTGGG GTATCTAATCC-3'	373
BVt	*Panthera tigris*	5'-CCAACAACCC TATGTCTTG-3'	5'-GCATAGTGGG GTATCTAATCC-3'	535
BVe	*Elephas maximus*	5'-AGAGTAGAAT ATAAACGGGAGGC-3'	5'-GCATAGTGGG GTATCTAATCC-3'	691

Table 4. Species- specific primer with their amplicon size. Analysis was done on SnapGene (v.8.1)

These species are forensically most relevant in India. Rhinoceros, elephant and tiger are most poached animals and cow is significant because of various state laws regarding cow slaughter. Post PCR these amplicons position in gel can easily be identified using ladder confirming the species in extremely short time span. Confirmatory result is obtained using one PCR reaction also eliminating the use of restriction enzymes, further reducing cost and time. Figure 3 summarizes the procedure for species identification using BV primers.

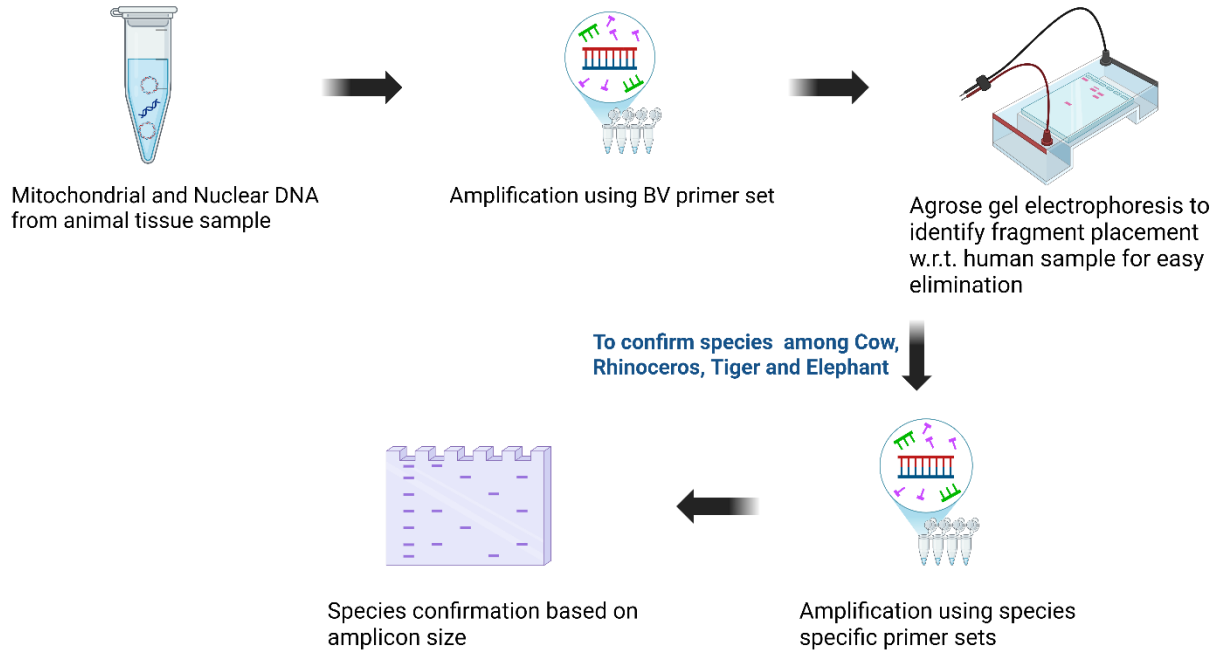

FIG-3. Step-by-step procedure for species identification using BV and species- specific (BVr, BVc, BVt, BVe) primer sets.

CONCLUSIONS:

Species identification is necessary in wildlife and illegal meat related crimes. Over the year there has been many tools being developed like PCR- RFLP, sequencing and by using various instrumental tools, but most of these techniques are costly, tedious, time consuming or require operational expertise in instruments. BV primer set can easily eliminate species on the basis of amplicon size observed in gel. Species specific BV primer sets will give amplicon of specific size unique to a species, reducing both cost and time. However, species specific primer of other species should be development as a single cocktail eliminating use of restriction enzyme and making species identification just one PCR reaction away. However, more species-specific primer sets should be developed for efficient use against all species.

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