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TECHNICAL NOTE

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Implication of Population Structure in the Resolution of Cattle Stealing Cases

ABSTRACT: Estimation of population subdivision using genetic markers shows that genetic differentiation in livestock and pet breeds is significantly higher than in human populations. Nevertheless, the influence of population substructure and sample size on match probability has not been extensively analyzed in domestic species. To evaluate the magnitude of the subpopulation effect on estimation of match probabilities in bovine robbery cases, we calculated and compared the match probabilities obtained from cattle breed databases using both real, adjudicated cases from the Buenos Aires Province (Argentina), as well as simulated data. While the Balding and Nichols' correction, when applied to the population database used in the case, produce a more conservative value favorable to the defendant, the match probabilities calculated using the simple product estimator produce a value favorable to the prosecution. We suggest an alternative procedure that can be used. The method consists of choosing the highest value from all match probabilities calculated from the database of each breed. This approach represents an intermediate and more accurate estimation of match probability, although it still produces a slight conservative value favorable to the defense.

KEYWORDS: forensic science, Bovine, cattle stealing cases, DNA interpretation, match probability, subpopulation effects

During the last decades, DNA profiling has become a common and well accepted methodology for pedigree or kinship analyses and paternity testing in livestock and wild species. Microsatellite typing has replace blood group and protein profiles in the assessment of cattle pedigrees and in the certification of semen and identity of embryos (1–5).

It is often difficult to obtain sufficient evidence to convict individuals suspected of illegal taking or trading of livestock and threatened species. Forensic methods, based on mtDNA and microsatellites analysis, can be used in these cases to identify the origin of the biological material. Molecular genetic techniques have assumed an important and growing role in the detection of illegal import, export, and hunting of endangered species, and theft of livestock.

Livestock robbery is a frequent occurrence in Argentina and in other undeveloped countries. Our lab usually receives evidence in robbery cases as diverse as meat, leather, bones and blood stains on clothes, butchering devices, and vehicles. In a previous study, we described a cow theft where DNA profiling was used as evidence to support the prosecutor's accusation in court (6). In that case, the thieves left the stolen animal's remains on the owner's farm (named as reference sample). The owner recognized them by the brand on the coat. Several meat and bone pieces (named as evidence samples) were then taken from alleged animals in the suspected butchery to compare them with the found remains.

There are two possible outcomes when DNA markers of evidence and reference samples are compared. First, if DNA markers do not match, they do not come from the same individual. Under these circumstances, there is no need for information about population frequencies of DNA markers. Second, if there is DNA match, evidence may be interpreted using the product rule to estimate the probability of a match by chance. However, objections have been raised to this statistical approach because of uncertainties. The first one, sampling uncertainty, is associated with the accuracy of the database. Was sample size sufficient? Was the sample representative of the population? The second one could be called the subpopulation problem or subpopulation uncertainty. Both types of uncertainty have been extensively discussed in human populations (7–10).

Previous works (11–13) have compared product rule estimates from differing human databases and found that the joint effect of both "sample" and "subpopulation" error may alter match probabilities by a factor of 10. NRC II recommended correction of subpopulation effects by the Balding and Nichols (14) method using θ values ranging from 0.01 to 0.03 (recommendations 4.1 and 4.2). Curran et al. (15), using hypothetical populations with fixed values of θ (0.01 and 0.03), extended these studies by comparing estimated to 'true match probabilities' to remove the effect of sampling variation. Their results support the conclusion that in humans subpopulation effects are slight.

The genetic uniformity of most human populations contrasts with what is observed in some other large mammals. Estimation of genetic subdivision using classical drift-based models and microsatellite markers showed that the average proportion of genetic differentiation among livestock and pet breeds was significantly higher than values observed in humans, for example, 7–11% for European cattle (16–18), 6% in pigs (19), 8% in horses (20), and 27% in dog (21). The degree of genetic differentiation among breeds indicates a relatively low gene flow between them. This is probably the result of the advent of breeder associations, herd books and breed standards, and the promulgation of breed barrier rules. Nevertheless, the influences of population substructure and sampling error in the match probability have not been extensively evaluated in domestic species.

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In the current study, we evaluate the magnitude of the subpopulation effect on the estimation of match probabilities for bovine robbery cases. To carry out this aim, we calculated and compared the match probabilities from different cattle breed databases (22) using both real data from cases from the Buenos Aires Province (Argentina) and simulated cases.

Materials and Methods

Studied Samples

The samples included on this report correspond to 15 cow thefts where DNA profiling was used as evidence. In these cases, after slaughter, the remains of the stolen animals were left by the thieves on the owner's farm. Several pieces of the remains were sequestered for use as reference material for comparisons with the evidence collected from the butchery.

DNA Extraction

Animals remains collected at the disposal site, including limbs and skin with hair, were retrieved and submitted to the laboratory by the Courts of Buenos Aires Province (Argentina). The meat was in a relatively high state of putrefaction, but DNA was extracted according to the modified methods suggested by Wagner et al. (23): 0.1 g of each sample (frozen at −80°C) were cut in small pieces with a scalpel, and suspended in 750 μL of digestion buffer (50 mM of Tris–HCL, 25 mM of EDTA, 20 mM of DTT, 2% of *N*-lauroylsarcosine) plus 30 μL proteinase K (10 mg/mL). The suspension was incubated overnight at 55°C. After incubation, 250 μL of 10 M ammonium acetate was added and the mix was centrifuged for 5 min at 14,000 g. The DNA was then precipitated with isopropanol, suspended in 200 μL of water, and then stored at −20°C until use. Genomic DNA concentration was quantified by comparison with standard DNA on 1% agarose gel after staining with ethidiun bromide.

Genetic Markers

DNA typing was performed by PCR using nine genetic markers. The microsatellites *ETH225*, *INRA023*, *BM1824*, *BM2113*, *SPS115*, *TGLA122*, and *TGLA227* were suggested by the international Society of Animal Genetics to be used for the International Comparison Test (http://www.isag.org.uk/, 24–29), while the microsatellites *MGTG7* and *TGLA53* were included in the FAO (Food and Agriculture Organization of The United Nations, 24,30) list for biodiversity studies (CaDBase Genetic Diversity in Cattle, http://www.projects.roslin.ac.uk/cdiv/markers.html).

PCR Amplification and Genetic Analysis of PCR Products

PCR was carried out in a total volume of 12.5 μL, containing 20 mM Tris–HCl (pH = 8.4), 50 mM KCl, 0.75–1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.75 U Taq polymerase (Invitrogen, Carlsbad, CA), 0.2–0.8 μM of each primer, and 10–20 ng of DNA. The cycling conditions were: a denaturation step of 2 min at 94°C, followed by 10 cycles of 1 min at 92°C, 45 sec at 57–62°C, and 50 sec at 72°C, and followed by 25 cycles of 1 min at 90°C, 45 sec at 57–62°C, and 50 sec at 72°C with a final elongation step of 15 min at 72°C. Variants were detected on 5% (19:1) polyacrylamide denaturing 50 cm gel by silver staining (31,32). Alleles were identified (bp size) by gel mobility comparison to previously typed DNAs that were included in the gel as standards.

Statistics

The likelihood ratio (LR) was calculated. LR is the ratio of the probability of observing the given DNA profile under two different hypothesis: (i) the evidence and reference samples originated from the same individual and (ii) the evidence sample originated from some other individual in the population. The probability that some other individual carried the same markers as the reference sample by chance is the match probability. It can be estimated as the genotype proportion in the randomly-mating, unstructured population. Suppose the profile A has alleles $A_{l,1}$, $A_{l,2}$ at locus l, being the their gene frequencies $p_{l,1}$ and $p_{l,2}$, respectively. If allele $A_{l,1}$ has the population frequency $p_{l,1}$, the genotypic frequency P_l at locus l is estimated as:

$$P_1 = \begin{cases} p_{l,1}^2, A_1 = A_2 \\ 2p_{l,1}p_{l,2}, A_1 \neq A_2 \end{cases}$$

Balding and Nichols (33) estimator takes population subdivision into account by incorporating the genetic correlation θ into the estimation of match probability:

$$P_l = \begin{cases} \frac{[2\theta+(1-\theta)p_{l,1}][3\theta+(1-\theta)p_{l,1}]}{(1+\theta)(1+2\theta)}, \mathrm{A}_1 = \mathrm{A}_2 \\ \frac{2[\theta+(1-\theta)p_{l,1}][\theta+(1-\theta)p_{l,2}]}{(1+\theta)(1+2\theta)}, \mathrm{A}_1 \neq \mathrm{A}_2 \end{cases}$$

Herein, we used a θ value of 0.1 calculated from our local gene frequency database (22).

The whole profile frequency P for l loci is estimated as:

$$P = \prod_l P_l$$

Simulation Analysis

We simulated a population of 500 individuals for each of five breeds (Aberdeen Angus, $N = 59$; Holstein, $N = 33$; Hereford, $N = 36$; Nelore, $N = 26$; Brahman, $N = 31$) using our local gene frequency database for these breeds and assuming Hardy–Weinberg and linkage equilibrium (22). After that, we determined match probabilities by the product rule, the method of Balding and Nichols, and by a novel method. We compared the match probability values obtained for each virtual animal in studies breeds.

Results and Discussion

When working with livestock, usually paternity and maternity probabilities are calculated rather than match probabilities, as their outcome affects the success of selection programs (1,2,4,5,34–39). However, recently, match probability between biological samples became an important issue in the field of traceability of animals and their products. Herein, we discuss its application to livestock robbery.

In 2001, we reported a case of livestock robbery where DNA profiling provided evidence for the detection of illegal hunting of cattle (6). Since that first report, we successfully solved several cases. For the present study, we selected seventeen pairs of biological evidence/reference samples corresponding to 15 livestock robbery cases, where the micro-satellite types of evidence and reference samples matched. The forensic biological materials were in a relatively high state of putrefaction, and the number of successfully typed loci varied from four to nine (Table 1).

TABLE 1—*Biological evidence in 15 livestock robbery cases, where the microsatellite types of evidence samples and reference samples matched. Match probabilities were calculated using the types observed in the forensic samples, by three different methods.*

Case	No. of loci typed	Product rule	Balding Nichols estimate	Aberdeen Angus	Holstein	Hereford	Nelore	Brahman
1	6	9,8E-10	1,3E-06	**2,8E-08**	1,5E-08	1,4E-10	6,4E-14	2,6E-15
2	4	4,8E-07	9,7E-05	3,4E-08	**5,3E-06**	2,0E-09	6,4E-09	3,6E-08
3	7	5,4E-12	5,3E-07	**5,7E-10**	1,8E-13	3,9E-12	1,8E-17	7,5E-15
4	7	1,7E-11	7,7E-08	1,9E-11	**8,4E-11**	6,0E-14	6,3E-16	1,2E-14
5	7	1,6E-12	2,8E-07	4,8E-14	**2,6E-12**	2,3E-15	9,3E-14	**2,6E-12**
6	9	1,2E-12	1,9E-09	**5,5E-10**	1,6E-10	4,4E-16	1,2E-19	6,3E-20
7	7	1,2E-07	6,8E-06	1,7E-11	1,3E-11	2,5E-10	**1,2E-07**	6,7E-09
8.1	8	1,7E-12	1,7E-08	9,8E-13	**1,0E-11**	7,1E-13	2,9E-16	2,3E-19
8.2	8	5,1E-14	1,6E-08	4,3E-15	**5,6E-13**	4,3E-15	5,0E-17	6,9E-19
9	6	7,7E-14	2,3E-06	1,5E-13	**1,7E-11**	1,7E-14	1,1E-17	3,6E-17
10	6	9,1E-10	1,4E-05	**8,2E-08**	3,9E-10	2,0E-10	1,3E-12	2,9E-11
11	5	1,8E-09	6,1E-06	1,1E-08	**1,9E-08**	3,9E-09	2,0E-14	1,1E-12
12	9	2,7E-20	3,1E-10	1,2E-20	1,7E-18	**2,9E-17**	8,6E-26	4,2E-22
13.1	6	1,7E-13	3,4E-07	5,2E-13	**1,8E-12**	1,7E-14	2,0E-15	2,4E-15
13.2	6	1,2E-12	1,4E-06	9,4E-13	**1,4E-10**	1,1E-11	2,8E-15	6,9E-15
14	7	1,1E-13	1,1E-06	**1,6E-10**	1,6E-12	6,5E-13	9,0E-17	5,6E-17
15	9	3,4E-16	7,1E-09	2,7E-15	**6,6E-15**	7,7E-16	1,5E-18	2,3E-20

Bold values represent the breed with highest match probability in each case.

The source of the population (i.e., breed origin) of biological evidence is usually unknown. For this reason, we start by considering the question of: "How dissimilar could be the estimation of the match probabilities between biological reference and evidence if different reference populations are considered"? The match probability was estimated using the types observed in the received forensic sample, by three different methods: (i) the biological evidence corresponded to a general unstructured population (defined here as bovines of Buenos Aires Province). In this method, the match probabilities are calculated using the simple product estimator; (ii) the θ correction (Balding and Nichols' estimator) was applied as if the population was divided into an unknown number of subpopulations, and (iii) match probabilities are estimated within each possible population using the product rule, as if the biological evidence could be assigned *a priori*.

Inspection of Table 1 shows that, as expected, the values obtained using the Balding and Nichols' correction were more conservative—by a factor of 2 to 10—than values calculated using the simple product rule in a nonsubstructured population. In other words, the Balding and Nichols' correction is conservative and favors the defendant. When match probabilities were estimated for each breed, the values varied from 3 to 10 orders of magnitude. In all cases, the largest difference in probability was observed between the taurine and cebuine breeds. In most of the cases the more conservative probability was obtained for the taurine breeds (Holstein and Aberdeen Angus), while in case number 7 it was observed in the cebuine breed Nelore. The probability values calculated using the individual breeds fall between the values calculated using the conservative theta correction and the product rule. These values are occasionally less than one order of magnitude different than the value calculated using the product rule.

Furthermore, we simulated a population of 500 individuals for each of the five breeds included in the paper using our local allele frequency databases for these breeds. We assumed Hardy–Weinberg and linkage equilibrium. We compared the match probability values obtained for each virtual animal in all pairs of breeds. The scatter plot representations of four of these comparisons (Nelore vs. Aberdeen Angus; Nelore vs. Holstein; Aberdeen Angus vs. Holstein and Nelore vs. Brahman) are detailed in Figs. 1*a*–1*d*.

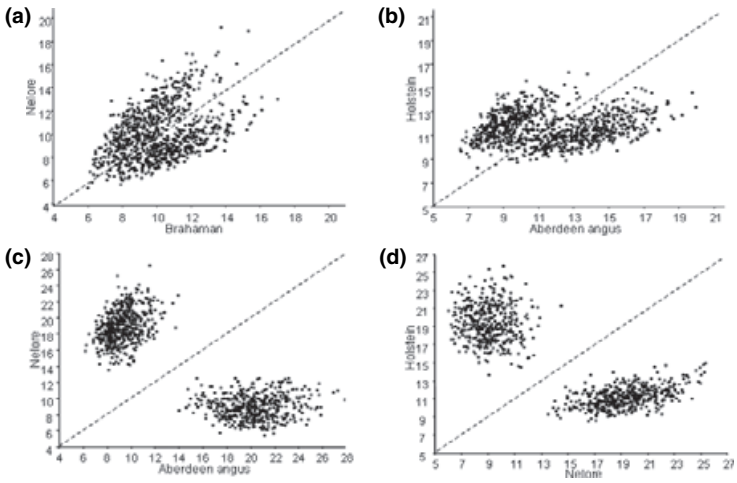

FIG. 1—*Scatter plots of pairwise match probabilities between two bovine breeds. (a) The abscissa and the ordinate give the estimated match probability in Nelore and Aberdeen Angus, (b) in Nelore and Hereford, (c) in Aberdeen Angus and Holstein, and (d) in Nelore and Brahman.*

When we compared a cebuine breed (Nelore, Brahman) against a taurine breed (Aberdeen Angus, Holstein, Hereford), the majority of the values lie between ±14 and ±18 orders of magnitude (Figs. 1*b* and 1*c*). Furthermore, the points were arranged into two discrete point's clusters. By contrast, when data from breeds within the same bovine type were compared, the patterns of differentiation were not as marked. However, the points of match probability still showed a considerable separation *g* covering more than five orders of magnitude (Figs. 1*a* and 1*d*).

In humans, the magnitude of the "subpopulation" error is not large, and some authors have suggested that it is important not to overemphasize it (10). Curran et al. (15) affirmed that subpopulation effects are mild because subpopulations and sampling uncertainty in humans are small in relation to the power of the evidence and may therefore be ignored.

It is not surprising that our results in bovine contrast considerably with patterns observed in humans, where the great majority of the points of match probabilities lie within ±10-fold when major groups are compared (review in 10). These results are in agreement with the fact that bovine, and other domestic animals, exhibit significant levels of population subdivision. In this sense, the Analysis of Molecular Variance with genetic markers show that variance among bovine breeds accounts for more than 10% of the total genetic variation. Thus, "subpopulation" error being larger for strongly subdivided populations than for more homogeneous ones, this type of uncertainty has a more considerable effect in domestic animals than it does in humans.

Balding and Nichols' correction when applied to the whole population was more conservative, favoring the defendant's hypothesis. On the other hand, the match probabilities calculated using the simpler product estimator produce results which are more favorable to the prosecution. In general, in a bovine robbery case context, the provenance of the biological evidence (meat and bone) is not known. We suggest the use of an alternative procedure in those cases, which consists in the calculation of a match probability based upon breed's database, selecting the higher value. Although this methodology is more complex and requires the knowledge of local populations' allele frequencies, it presents an intermediate and more accurate estimation. Furthermore, the choice of the populations with the higher value of match probability was slight more conservative in favor of the defense.

Currently, the availability of multiple polymorphic DNA genetic markers has created the opportunity to uses individual genotype information to determine the population origins of individuals or samples. In addition, several assignment tests and computer packages have been reported to achieve this aim. Some of them are based on computation of the match probability of the individual in each population (16,40–43). These algorithms could be useful in a forensic context.

Finally, the present analysis can also be very useful in agriculture for the traceability of animals or animal products (e.g., assignment of a carcass, an embryo, sperm to a breed, or milk sample). In this sense, further studies are necessary to increase our knowledge on the dynamics and structure of local bovine populations.

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