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ORIGINAL ARTICLE

Comparative evaluation of alternative batteries of genetic markers to complement autosomal STRs in kinship investigations: autosomal indels vs. X-chromosome STRs

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Abstract Kinship investigations such as paternity are currently solved using sets of (commercially available) highly polymorphic autosomal short tandem repeats (STRs), which lead to powerful likelihood ratios (LR). Still, some difficult cases arise whenever the kinship is much more remote or if the alternative hypotheses are not correctly formulated due to the lack of information (for e.g. there is an unknown relationship between the alleged and the true fathers). In these situations, beyond the routinely used marker set, laboratories usually enlarge the number and/or the type of markers analysed. Among these, autosomal indels and X-chromosome STRs have gained popularity. The aim of this study was to compare the results obtained after complementing an initial set of autosomal STRs with indels or with X-chromosome-specific STRs in simulated paternity cases where the alleged father is a close relative of the real one. Results show that in paternity cases where a low number of incompatibilities are observed, the best strategy is to increase the number of autosomal STRs under analysis. Nevertheless, if these are not available, our study globally shows that in father–daughter duos, a set of 12 X-STRs is more advantageous than 38 highly diverse autosomal biallelic markers. Additionally, the usefulness of X-STRs was also evaluated in cases where only a close relative of the alleged parent (father or mother) is available for testing. For those situations where these markers have the power to exclude, strong LR values are obtained. In the remaining cases, LRs are usually weak and sometimes the results are more likely under the wrong kinship hypothesis.

Keywords Indels · Deficiency cases · X chromosome · Likelihood ratio · Paternity · Maternity · Kinship

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Introduction

The occurrence of Mendelian incompatibilities between true father and child is not an uncommon situation due to mutation or silent alleles. In paternity testing routine, for some cases, ambiguous statistical results are obtained, with few incompatibilities associated with high likelihood ratios (LR) for the remaining loci [1]. A large proportion of these cases arise from unknowingly testing, as alleged father, a close relative of the real father (a brother, the father or a son).

Usually, when the statistical evidence obtained is considered insufficient, additional markers are analysed. Insertion/deletion (indels) and single nucleotide polymorphisms (SNPs) have been suggested as markers of choice to include in these expanded batteries [2–4], as they are much less prone to mutation than short tandem repeats (STRs) [5]. On the other

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hand, X-chromosomal markers can also be useful in such cases [6] due to their unique transmission properties. Indeed, X-STRs have emerged as an asset in certain genealogical problems, not only complementing and completing autosomal marker information but also by solving certain kinship studies irresolvable with autosomal markers, as in some cases of "half-siblings", "avuncular" or "grandparent-grandchild" (e.g. [7–10]).

The analysis of the X chromosome can be very useful to exclude daughters' paternity when the alleged father is the father (or a son) of the real one because, from the specific X-chromosomal point of view, a paternal grandfather and his granddaughter (or a pair paternal half-brother–sister) are such as unrelated. Yet, if the alleged father is a brother of the true one, the analysed individuals are "paternal uncle–niece", for which case the probability of sharing one pair of X-alleles due to familial inheritance (identical-by-descent, or IBD, alleles) is the same as for autosomes: 50 %. Thus, the probability of finding no incompatibilities is similar when using X-chromosomal markers or autosomes.

In this work, data collected from families are used to illustrate the expected contribution of a set of 38 autosomal indels and of 12 X-chromosome-specific STRs in two types of kinship analysis.

First, the performance of low-mutating biallelic markers vs. that of X-specific STRs was compared when, based on the results from 15 autosomal STRs, the LR was weak, raising the issue that the analysed individual could actually be a close relative (brother, son or father) of the true father.

Second, the utility of the 12 X-STRs included in the Argus X-12 kit (Qiagen) was evaluated in cases where the informative power of the X-chromosome markers is expected to be higher than that for equally polymorphic autosomal ones [11, 12], namely in "paternity/maternity" cases where the alleged father/mother is not available for typing.

Materials and methods

Sample selection

Samples from 123 unrelated duos linked by different second degree pedigrees (Table S1) were collected and used to simulate the following:

Analysis 1 Paternity cases where a close relative of the biological father is unknowingly tested as the alleged one. With this purpose, pairs of "avuncular", "grandparent-grandchild" and "half-siblings" were selected. Based on autosomal STR, autosomal indel and X-STR results, LRs were computed comparing the hypotheses "father–child" vs. "unrelated"

Analysis 2 Situations where the alleged parent (father or mother) is not directly available for testing and, in this case, pairs of "paternal grandmother–granddaughter" and "maternal aunt–nephew/niece" were selected. LRs were computed based on both autosomal and X-chromosomal STR results.

Genotyping protocols

The selected duos were typed for the 15 STRs included in the AmpFlSTR® Identifiler™ PCR Amplification Kit (Applied Biosystems) and for the 12 X-STRs from the Investigator Argus X-12 Kit (Qiagen) following the protocols included in the user's manual of the manufacturers. The 38 autosomal indels were typed as described in Pereira et al. [13].

Likelihood ratio calculations

The Familias pedigree analysis software [14] was used to calculate LRs for autosomal markers. For X-STRs, joint genotypic probabilities were calculated as described by Pinto et al. [10].

Calculations have been based on previously described allele frequencies for autosomal STRs [15] and SNPs in the European population [12]. Mutation rates were those from the AABB Report [16] for STRs. For the indel markers, a mutation rate of $\mu=2.3\times10^{-9}$ [17] was used.

For (biallelic) indels, father–child incompatibilities are always associated to opposite homozygote genotypes whenever duos are analysed (undoubted mother not available for testing), and therefore, apart from mutation, they can also be explained by the presence of a silent allele. In this study, a frequency of $s=0.001$ was used, assuming the most conservative value described [18–20].

For the 12 X-STRs, haplotype frequencies were obtained by pooling Portuguese (see Table S2) and German population data [21], after confirming that no significant differences exist for these haplotypes between the two population samples.

The statistical results for the 12 X-STRs included in the Investigator® Argus X-12 kit were obtained by considering a mutation model similar to the one used by the Familias software [14], where the gain or loss of one repeat occurs with a probability of 10^{-3}, multiplying each of the required mutational steps by the factor 0.1 to conform with the kinship hypothesis. Moreover, a mutation rate of 2.3×10^{-9} [17] was considered for the cases where changes involved a non-multiple of the repeat motif number.

Results and discussion

The importance of using different genetic systems to extend the routine battery of STR markers in some difficult cases of paternity investigation has been thoroughly emphasised (see

[2–4, 6–9], for example). Aiming to evaluate the contribution of alternative markers for complementing the most widely used autosomal STRs, by using real family data, we have elaborated different kinship hypotheses that can potentially arise in a laboratory performing casework, considering two types of paternity investigation scenarios.

Analysis 1. Paternity cases where the alleged father is a close relative of the real one

Ambiguous results may arise from unknowingly testing, as alleged father, a close relative of the real one (e.g. his brother or father). In order to illustrate the expected results when using a set of 15 STRs in these situations, Magalhães et al. [22] and Pinto et al. [2] analysed 100 duos of real second degree relatives, showing that results were inconclusive in 33 % of the cases, for five of which, the results being more likely under the (false) hypothesis of paternity. When not considering the possibility of silent alleles, most inconclusive cases were resolved with the additional typing of indels (case 1.1 in Table S1). However, whenever no incompatibilities were found with the indel markers, the results were always more likely under the "wrong" hypothesis of paternity. In the end, the results were inconclusive in 9 % of the cases [22]. This value increased to 82 % when taking into account the possibility of silent alleles (Table S5 in [2]). When STR and indel results were combined, 25 % of the cases remained inconclusive. Thus, if we cannot exclude the possibility of silent alleles, any indel incompatibility observed between duos of "alleged father–child" has an impact similar to the one obtained with STRs. This way, the claimed main advantage of the use of biallelic markers in paternity investigations—its low mutation rate—is lost.

With this first analysis, we intended to estimate the informative power of 12 STRs included in the kit Investigator® Argus X-12 (Qiagen) in paternity testing with daughters (and second-degree relatives) when the LR obtained with autosomal markers is low. LR values were calculated assuming the relationship "father–daughter" against "unrelated". Such as in other studies [4], it was considered that a LR between 10^{-4} and 10^{4} is inconclusive in paternity tests dealing with samples of good quantity/quality. Additionally, paternity indexes calculated for "avuncular", "grandparent-grandchild" and "half-siblings" duos were compared when using 15 autosomal STRs, plus either a set of 38 autosomal indels or a set of 12 X-STRs.

Complementing inconclusive STR results with X-chromosomal STRs

Ten pairs of samples linked by a known pedigree were used to reproduce paternity tests involving close relatives (see case 1.2 in Table S1). Due to the (non)transmission of X-chromosomes, the kinship "father–son" has not been considered. Still, in order to enlarge the sampling, indistinguishable relationships [10], belonging to the same X-chromosomal class, were also pooled; specifically, "maternal grandmother-grandson" and "maternal grandfather–granddaughter" were used to simulate "paternal uncle-niece" cases.

Examining the results obtained with autosomal STRs (Table 1; full data are included in supplementary Table S3), six out of the 10 cases presented LR values that are compatible with a paternity exclusion. In the remaining cases, the LR value obtained for the 15 autosomal STRs would justify an additional trial with another set of markers.

The set of 12 X-STRs was informative, favouring the hypothesis of unrelatedness, except in one case for which paternity was excluded using Au-STRs, but the X-STR results were much more likely under the paternity hypothesis, and no incompatibilities were found.

Complementing inconclusive STR results: X-chromosomal STRs vs. indels

In six cases, autosomal STRs were complemented with indels, or with X-chromosomal markers (see case 1.3 in Table S1). The results obtained (Table 2; full data are included in supplementary Table S4) show that X-STRs were more efficient than indels to exclude paternity, except in one case where no incompatibilities were found for both indel and X-STR markers, being the results stronger in favour of the (false) hypothesis of paternity for the 12 X-STRs.

Analysis 2. Parentage investigations when the alleged parent is not available

With this second analysis, we intended to estimate the informative power of 12 STRs included in the kit Investigator® Argus X-12 (Qiagen) in parentage investigations when the alleged parent is not accessible for testing, and it is necessary to analyse close relatives. Distinct cases were considered whenever the informative power of the X-specific markers is known to be higher than that for equally polymorphic markers on autosomes. For each case, LR calculations were computed

Table 1 Results obtained when using the 12 X-STRs and 15 Au-STRs using (real) pairs of second degree relatives

No. of duos	15 Au-STRs	12 X-STRs	Au-STRs × X-STRs
5	Against paternity	Against paternity	Against paternity
3	Inconclusive	Against paternity	Against paternity
1	Inconclusive	Inconclusive	Inconclusive
1	Against paternity	Favouring paternity	Favouring paternity

Table 2 Comparison of the results obtained with the autosomal and X-chromosomal markers using (real) pairs of second degree relatives

No. of duos	15 Au-STRs	38 Indels	Au-STRs × Indels	12 X-STRs	Au-STRs × X-STRs
1	Inconclusive	Inconclusive	Inconclusive	Against paternity	Against paternity
3	Against paternity	Inconclusive	Against paternity	Against paternity	Against paternity
1	Against paternity	Against paternity	Against paternity	Against paternity	Against paternity
1	Against paternity	Inconclusive	Inconclusive	Favouring paternity	Favouring paternity

considering the following hypotheses: the individuals are assumed related as self-declared ("paternal grandmother–granddaughter", for example) or as unrelated.

Paternity investigations

In paternity cases using X markers, analyses can be performed by typing the mother, one daughter, one (full-) sister or one (full-) brother of the alleged father, so that the kinships paternal "grandmother–granddaughter", "half-sisters", "aunt–niece" and "uncle-niece", respectively, are in question.

For the cases "grandmother–granddaughter" and "half-sisters", the expected probabilities of sharing IBD X-alleles are different from those for autosomal alleles. Indeed, any duo of "paternal grandmother–granddaughter" and "paternal half-sisters" will certainly share one pair of IBD X-alleles, unless it mutates, but it is equally likely that they share one or no pair of IBD autosomal alleles. In fact, the kinships "paternal grandmother–granddaughter" and "paternal half-sisters" are the only ones where incompatibilities with (fully codominant) Mendelian rules of transmission can be found even when only duos are analysed, which lead to powerful statistical results.

Statistical results were calculated considering eight pairs of paternal "grandmother–granddaughter" (see case 2.1 in Table S1) for both 15 autosomal STRs and 12 X-STRs, showing that the results obtained with X-STRs are much more powerful than those obtained with autosomes (Table S5). For comparison purposes, calculations were also performed considering ten real pairs of paternal aunt–niece (see case 2.1 in Table S1 and Table S6), and, as expected, the differences between the values obtained with autosomal or X-chromosomal STRs revealed to be much smoother.

Thus, when the alleged father is not available for testing, X markers should be privileged whenever his mother (or one daughter) is accessible for typing. On the other hand, when such relatives are unavailable and the X-chromosomal transmission is not broken, the usefulness of X markers is expected to be comparable as that of autosomes.

Maternity investigations

For X markers, when maternity is in question, it is possible to resort to any direct parent of the alleged mother since the chain of X-genetic transmission is not interrupted in the maternal kinships "grandparent-grandchild", "half-siblings" and "avuncular". For the pedigrees "half-siblings" and "grandparent-grandchild", the usefulness of the X markers is the same as of the autosomal counterparts, because the probability of sharing or not one pair of IBD X-alleles is also equally likely. Nevertheless, it should be highlighted that in the pedigree "maternal uncle–nephew/niece", the probability of sharing one pair of IBD X-alleles is even lower (¼) than that accomplished considering autosomes (½).Statistical results for such cases are presented in supplementary material Table S7 for eight real duos. The LR values were weak for both autosomal and X-chromosomal STRs, and in three cases, X-STR results were more likely under the wrong hypothesis of unrelatedness.

On the other hand, higher probabilities of sharing IBD X-alleles relatively to autosomes are only achieved in "maternal aunt–niece/nephew" cases, in which individuals share one pair of IBD X-alleles with probability ¾ (being 1/2 for autosomes). The LR was computed for 20 real pairs of "maternal aunt–nephew/niece" (see case 2.2 in Table S1), and the results are depicted in Table S8. As expected for the number of analysed markers [23], the majority of the results are not very powerful when both alternative kinship hypotheses are a priori equally likely.

Combining the information of both autosomal and X-STRs, the final LR was over 10^4 in a single case.

Conclusion

Considering the paternity situations where the alleged father is a close relative of the real one, we can conclude that both sets of markers (autosomal indels and X-STRs) can be useful to exclude false fathers, when the evidence from the routine autosomal STR battery is considered as insufficient.

The weight of the achieved LR results is although much weaker for indels than for X-STRs. Indeed, X-chromosomal specific markers allow excluding false fathers with higher confidence. However, caution should be taken when no incompatibilities are detected since, under these circumstances, the (false) hypothesis of paternity may be strengthened when considering unrelatedness as the second hypothesis for computing the LR.

Regarding paternity investigations (with daughters) where the alleged father is not accessible for testing, it was concluded that if his mother (or one daughter) is accessible, powerful results are expected for the currently available X-STR markers. In the remaining situations, there is no significant difference between autosomal or X-chromosomal results.

Regarding maternity investigations where the alleged mother is not accessible for testing and one of his close relatives is analysed, it was concluded that X markers should be chosen first than autosomes only if a sister is available for testing. Nevertheless, the fact that just four X-chromosome "marker groups" are available seriously limits their power to solve most of these cases without a complementation by autosomal markers.

In summary, for those situations where X-chromosome markers have the power to exclude, high LR values are obtained. In the remaining cases where relatives are involved, LRs are usually low, and sometimes the results are more likely under the wrong kinship hypothesis.

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