Rapidly Mutating Y Chromosomal STRs in Deep-Rooted Endogamous Pedigrees

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Research Article

Keywords: Endogamous, Punjab, Pakistan, deep rooted pedigrees, RM Y STRs, Mutation rates

Posted Date: October 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-919302/v1

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Abstract

Y-chromosome short tandem repeat polymorphisms (Y-STRs) are important in many areas of human genetics. Y chromosomal STRs being normally utilized in the field of forensic exhibit low haplotype diversity in endogamous populations and fail to discriminate among male relatives from same pedigree. Rapidly mutating Y-STRs (RM Y-STRs) have been paid much attention in last decade. These 13 RM Y STRs have high mutation rates (>10-2) and have considerably higher haplotype diversity and discrimination capacity than conventionally used Y-STRs showing remarkable power, when it comes to differentiation in paternal lineages in endogamous populations. Previously, we have analyzed 2–4 generation, 99 pedigrees covering 1568 pairs of men covering 1–6 meioses from all over Pakistan and 216 male relatives from 18 deep rooted endogamous Sindhi pedigrees covering 1-7 meioses. Here we are presenting 861 pairs of men from 63 endogamous pedigrees covering 1-6 meioses from Punjab population of Punjab, Pakistan. Mutations were frequently observed at DYF399 and DYF403 while no mutation was observed at DYS526a/b. The rate of differentiation ranged from 29.70% (first meiosis) to 80.95% (fifth meiosis) while overall (1 to 6 meiosis) differentiation was 59.46%. Combining previously published data with newly generated data, an overall differentiation rate was 38.79% based on 5176 pairs of men related by 1–20 meioses, while Y-filer differentiation was 9.24% based on 3864 pairs. Using father-son pair data from the present and previous studies, we also provide updated RM Y-STR mutation rates.

Introduction

Y-chromosomal microsatellites or short tandem repeats (Y-STRs) play an important role within forensic genetics1–3. Most commonly, Y-STRs are used to determine the male component of DNA mixtures when a high female background is present 4,5, or to determine paternal relationships between male individuals 6–8. Currently, a large and growing reference databases exists for estimating Y-STR haplotype frequencies among worldwide human populations (e.g. http://www.yhrd.org or http://usystrdatabase.org/). Commercially available Y-STR kits (Yfiler, Powerplex 23 and Yfiler Plus) are valuable, but they have some limitations for their use in forensic casework. Overall, the haplotype diversities (HD) of these sets of Y STRs are good for outbreeding populations, usually at 0.995 and higher 9,10. However, the ability to discriminate between individuals is much lower than that of the autosomal STRs. In endogamous populations or populations which run into size contraction followed by quick expansion recently11,12, or have particular cultural practices such as patrilocality 13,14, the currently used Y-STR panels provide limited resolution due to the overall reduced Y-chromosome diversity.

The major drawback of currently used Y-STRs is that they are unable to exclude close or distant patrilineal relatives of the suspect from having deposited the biological material instead of the suspect himself. These STRs maybe helpful in those cases where close or distant male relatives may be involved because of their relatively low mutation rates of only a few mutations per thousand generations per locus 6,8,15,16.

In 2010, Ballentyne et al 16 reported mutation rates of 186 Y STRs in 2000 father-son pairs. This study identified 13 Y-STR markers with markedly higher mutation rate of about 10^2 and termed them rapidly mutating (RM) Y-STRs.

In the current study, we have provided empirical evidence of the ability of the 13 RM Y-STRs for improving paternal lineage resolution by analysing 861 pairs of men from 63 endogamous pedigrees covering 1-6 meiosis from Punjab, Pakistan.

Materials And Methods

Pedigree Samples and DNA Extraction

Initially 63 pedigrees 1-4 generations consisting of 861 pairs of men were located in different areas of Punjab Pakistan. 327 Blood samples were collected from 63 families. Pedigree of these family members was generated according to their oral records, which were later on confirmed with National Identity Card (NIC). In case of any confusion family registration certificate (FRC) was applied using NADRA (National Database and Registration Authority) online paid services. All participants gave their informed consent either orally (in case they could not write) or in writing, after the study aims and procedures were carefully explained to them. This study was approved by the ethical review board of University of Health Sciences Lahore Pakistan and was in accordance with the principles of the Declaration of Helsinki made at 64th WMA General Assembly, Fortaleza, Brazil, in October 2013. All blood samples were stored at -20 °C before DNA extraction. DNA was isolated from blood using ReliaPrep™ Blood gDNA Miniprep System (Promega, Madison, USA,) according to the manufacturer's instructions. The quantities of extracted DNA samples were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington DE, USA) and the final concentration of DNA was diluted to 1 to 2 ng/μl.

PCR amplification and genotyping

PCR co-amplification of thirteen rapidly mutating Y-STR loci (DYF387S1, DYF399S1, DYF403S1ab, DYF404S1, DYS449, DYS518, DYS526ab, DYS547, DYS570, DYS576, DYS612, DYS626 and DYS627) were performed in a 5-dye fluorescence-based multiplex reaction using the RM-Yplex assay 17. From 1 to 2 ng of the target DNA was amplified according to the manufacturer's recommended protocol. Thermal cycling was
conducted under the following conditions: 95°C for 10 min; 20 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 60 s; and a final extension of 72°C for 45 min. All loci were amplified in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). Amplified products were analyzed regarding GS600 LIZ size standard and Allelic Ladder using an ABI 3500 genetic analyzer (Applied Biosystems, Foster City, CA) with the POP-6™ polymer (Life Technologies). Samples were analysed using GeneMapper®ID-X software version 1.2 at a threshold of 50 rfu.

**Statistical analysis**

Rate of differentiation among male relative pairs were calculated as number of differentiated pairs of relatives by one or more Y STRs divided by the total number of male relative pairs on that particular meiosis or degree of relationship (i.e., pair members separated by 1–20 meiosis). Total numbers of mutations observed dived by number of father-son tested were used to calculate the mutation rates and binomial standard deviation was used to calculate the mutation rates 95% confidence intervals (CI) which is available via [http://statpages.org/confint.html](http://statpages.org/confint.html). Haplotype diversities were calculated as

\[
\left(\frac{n}{n - 1}\right) \left(1 - \sum f_i^2\right)
\]

where \(n\) is the number of samples, and \(f_i\) the frequency of the \(i\)th haplotype.

**Results And Discussion**

**Non-paternity Issues**

The non-paternity events are common in pedigrees and these events were also observed in Pakistani population dataset. To overcome this issue, we split the pedigrees accordingly. In some pedigrees only one individual was involved in non-paternity. After measuring the genetic distance that individual was removed from pedigree. Individuals with mutation event on \(\geq 4\) STRs were removed. This threshold was derived from our previous observations based on >2000 father-son pair carrying mutations at three Y-STRs; while none of these showed mutations at more than four Y-STR markers.\(^8,18\).

**Male relative differentiation from current study**

We have analysed the set of 13 RM Y STRs which was described previously\(^6,15,16,19,20\) in 327 Punjabi males belonging to 63 pedigrees 2-4 generations, representing a total of 861 pairs of men related by 1-6 meiosis. Among these 861 pairs, 512 (59.46%) were differentiated by at least one of the 13 RM Y-STR markers (Table 1). More specifically, of the 135, 171, 155, 204, 168, and 28 male relative pairs were separated by 1 (father/son), 2 (brother, grand-fathers/grand-son), 3 (great grand-fathers/ great grand-son, uncles/nephew), 4 (cousin, grand uncle/ grand-nephew), 5 (first cousin once removed) and 6 (second cousin) meioses, respectively. The RM Y-STR set separated 29.7%, 39.76%, 60%, 68.13%, 80.95%, and 78.57%, pairs respectively. We also noted an increase in relative pair differentiation when there is increased number of meiosis (Table 1). Theoretically, increased differential power of STRs was expected because more chance of mutation events happens at independent meiosis.

| Relationship                  | Total Number of Pairs | Pairs separated by one or more RM Y STRs marker (%) |
|-------------------------------|-----------------------|-----------------------------------------------------|
| Father/Son                    | 135                   | 40 (29.70%)                                         |
| Grandfather/Grandson          | 67                    | 28 (41.80%)                                         |
| Brother/Brother               | 104                   | 54 (51.92%)                                         |
| Uncle/Son                     | 147                   | 87 (59.18%)                                         |
| Great Grand Father/ Great Grand son | 8                    | 6 (75%)                                             |
| 1st Cousin/1st Cousin         | 190                   | 129 (67.90%)                                        |
| Grand Nephew/ Uncle           | 14                    | 10 (71.42%)                                         |
| 1st Cousin                    | 168                   | 136 (80.95%)                                        |
| 2nd cousin                    | 28                    | 22 (78.57%)                                         |
| Total                         | 861                   | 512 (59.46%)                                        |

We observed that maximum number of pairs were differentiated based on mutation in multiple markers. 512 pairs were differentiated by at least one of the 13 RM Y-STR markers, 392 (76.56%) pairs were differentiated by multiple markers. More specifically, of the male relative pairs
separated by 1 (father/son), 2 (brother, grandfather/grandson), 3 (great grandfather/great grandson, uncle/nephew), 4 (cousin, grand uncle/grandnephew), 5 and 6 meiosis; the 13 RM Y-STR markers set separated 75%, 71.95%, 82.80%, 79.14%, 72.05%, and 81.81%, pairs respectively. Overall, 76.56% were differentiated by multiple markers. Pairs separated by single-copy markers were only 23.43% (Table 2).

| Relationship                      | Meioses | Number of pairs | Number of pairs separated by 13 RM Y-STRs | Pairs separated by Multicopy markers (%) | Pairs separated by single-copy markers (%) |
|-----------------------------------|---------|----------------|------------------------------------------|------------------------------------------|------------------------------------------|
| Father/Son                        | 1       | 135            | 40                                       | 30 (75%)                                 | 10 (25%)                                 |
| Grand Father/Grandson & Brother/Brother | 2       | 171            | 82                                       | 59 (71.95%)                              | 23 (28.05%)                              |
| Great Grand Father/Great Grandson & Uncle/Son | 3       | 155            | 93                                       | 77 (82.80%)                              | 16 (17.20%)                              |
| 1st cousin/1st cousin & Grand Nephew/Uncle | 4       | 204            | 139                                      | 110 (79.14%)                             | 29 (20.86%)                              |
| 5th meioses                       | 5       | 168            | 136                                      | 98 (72.05%)                              | 38 (27.95%)                              |
| 6th meioses                       | 6       | 28             | 22                                       | 18 (81.81%)                              | 4 (18.19%)                               |
| Overall                           | 861     | 512            | 392 (76.56%)                             | 120 (23.43%)                             |

Male relative differentiation from the current and previous studies

The differentiation rate of 29.7% using 13 RM Y-STRs for 135 pairs was fairly consistent with those in published in previous studies \(^6,15,20\). Merging previously published data and newly generated data showed an overall rate of 26.55% from a total of 2990 pairs (Table 3). In current study, we provide an update on male relative differentiation for father-son pairs (n = 726) relative to the previous studies (total n = 1460)\(^18-20\). Combing RM Y-STR data (Table 3), males separated by 2, 3, and 4 meioses are differentiated by 43.69%, 52.86%, and 63.22% which are based on 801, 507, and 533 pairs, respectively. For males separated by 5 and more meioses, we added data for 196 pairs (5th meioses = 168, 6th meioses = 28).
Table 3
Combined male relative differentiation rates from the current and previous studies for the RM Y-STR set and the Y-filer Y-STR set

| Number of meioses separating relative pairs | Number of relative pairs analyzed for RM Y-STRs | Number of male relative pairs separated by one or more of 13 RM Y-STRs (%) | Number of relative pairs analyzed for Y-filer Y-STRs | Number of male relative pairs separated by one or more of 17 Y-filer Y-STRs (%) |
|---------------------------------------------|-----------------------------------------------|------------------------------------------------|-----------------------------------------------|------------------------------------------------|
| 1                                           | 2990                                          | 794 (26.55%)                                    | 2474                                          | 119 (4.81%)                                    |
| 2                                           | 801                                           | 350 (43.69%)                                    | 590                                           | 62 (10.51%)                                     |
| 3                                           | 507                                           | 268 (52.86%)                                    | 333                                           | 51 (15.32%)                                     |
| 4                                           | 533                                           | 337 (63.22%)                                    | 318                                           | 73 (22.95%)                                     |
| 5                                           | 231                                           | 178 (77.05%)                                    | 63                                            | 23 (36.50%)                                     |
| 6                                           | 76                                            | 56 (73.68%)                                     | 48                                            | 18 (37.50%)                                     |
| 7                                           | 14                                            | 4 (28.57%)                                      | 14                                            | 1 (7.14%)                                       |
| 8                                           | 7                                             | 5 (71.43%)                                      | 7                                             | 1 (14.28%)                                      |
| 9                                           | 1                                             | 1 (100%)                                        | 1                                             | 1 (100%)                                        |
| 10                                          | 7                                             | 6 (85.71%)                                      | 7                                             | 4 (57.14%)                                      |
| 11                                          | 6                                             | 6 (100%)                                        | 6                                             | 3 (50%)                                         |
| 12                                          | 0                                             | 0                                               | 0                                             | 0                                               |
| 13                                          | 2                                             | 2 (100%)                                        | 2                                             | 1 (50%)                                         |
| 14                                          | 0                                             | 0                                               | 0                                             | 0                                               |
| 15                                          | 0                                             | 0                                               | 0                                             | 0                                               |
| 16                                          | 0                                             | 0                                               | 0                                             | 0                                               |
| 17                                          | 0                                             | 0                                               | 0                                             | 0                                               |
| 18                                          | 0                                             | 0                                               | 0                                             | 0                                               |
| 19                                          | 0                                             | 0                                               | 0                                             | 0                                               |
| 20                                          | 1                                             | 1 (100%)                                        | 1                                             | 0                                               |
| Total                                       | 5176                                          | 2008 (38.79%)                                   | 3864                                          | 357 (9.24%)                                     |

Hopefully as more data is gathered for RM Y-STRs, these differentiation rates will become more robust, especially for 2nd degree male relatives (beyond 4th, 5th and 6th meioses).

We have shown this with the differentiation of father-son pairs from the first to the present study. In first study Ballantyne et al. 16, the rate of differentiation for father-son was 70% based on 20 pairs. In a follow up Ballantyne et al. study 15, this rate was decreased to 49% and number of father son pairs were 39. The rate of differentiation in the following Ballantyne et al. study 21 was further decreased to 27%, where the number of pairs were 2327. In another follow-up Adnan et al. study 6, the findings were similar with 24% differentiation rate in 428 pairs. In Rakha et al. study 20, we analysed 49 pairs with 20.40% differentiation rate, while 29.70% of differentiation rate was observed for 135 pairs here. In contrast, Y-filer differentiated 4.81%, 10.51%, 15.32% and 22.95% of related males by 1–4 meioses based on 2474, 590, 333, and 318 pairs studied so far, respectively (Table 3). Nonetheless, conventional Y-STRs tends to have lower mutation rates 8,18 and 9.24% of all related males from the combined studies were differentiated with Y-filer as opposed to 38.79% with RM Y-STRs.

**Mutation rate estimates from father-son pairs**

We also have calculated the mutation rates of these rapidly mutating Y-STRs which are based on 135 father-son pairs extracted from the 63 pedigrees, and combined this data with previous studies 6,15,16,20,21, where the same rapidly mutating Y-STRs panel was implemented for other father-son pairs (Table 4). The mutation rates ranged from 0 (0- 2.70 X 10^{-2}) for DYS526a or DYS526b to 1.778 X 10^{-1} (1.174 X 10^{-1} to 2.529 X 10^{-1}) for DYF399S1. The average mutation rate across all 13 RM Y-STR markers was 2.91 X 10^{-2} based on a total number of 59 mutations from
2025 meioses. Combining this new data with previously available data, the mutation rates ranged from $1.90 \times 10^{-3}$ ($7.0 \times 10^{-4}$ to $4.0 \times 10^{-3}$) for DYS626a to $7.45 \times 10^{-2}$ ($6.58 \times 10^{-2}$ to $8.39 \times 10^{-2}$) for DYF399S1. These currently estimated mutation rates are most reliable for the 13 RM Y-STR markers, given the underlying number of meiosis ranging from 2949 (DYS570) to 3327 (DYF387S1) between markers. The average mutation rate across all 13 RM Y-STR markers was $1.84 \times 10^{-2}$ ($1.72 \times 10^{-2}$ - $1.96 \times 10^{-2}$) based on a total number of 878 mutations from 47731 meioses.

### Table 4: RM Y STR mutation rates obtained from father-son pairs in the present and previous studies

| Locus   | No. of mutations | Samples | Current Study Mutation Rates (95% Confidence Interval) | Combined $^{3,5,20,21}$ No. of Mutations Total Samples Mutation Rates (95% Confidence Interval) |
|---------|------------------|---------|--------------------------------------------------------|-------------------------------------------------------------------------------------------------|
| DYS576  | 4                | 135     | $2.06 \times 10^{-3}$ ($8.1 \times 10^{-3}$ - $7.41 \times 10^{-3}$) | 44 3250 $1.33 \times 10^{-2}$ ($9.9 \times 10^{-3}$ - $1.81 \times 10^{-2}$) |
| DYF399S1| 24               | 135     | $1.778 \times 10^{-1}$ ($1.774 \times 10^{-1}$ - $2.529 \times 10^{-1}$) | 247 3317 $7.45 \times 10^{-2}$ ($6.58 \times 10^{-2}$ - $8.39 \times 10^{-2}$) |
| DYF387S1| 5                | 135     | $3.70 \times 10^{-2}$ ($1.21 \times 10^{-2}$ - $8.43 \times 10^{-2}$) | 47 3327 $1.41 \times 10^{-2}$ ($1.64 \times 10^{-2}$ - $1.87 \times 10^{-2}$) |
| DYS570  | 2                | 135     | $1.48 \times 10^{-3}$ ($1.8 \times 10^{-3}$ - $5.25 \times 10^{-3}$) | 32 2949 $1.22 \times 10^{-2}$ ($7.40 \times 10^{-3}$ - $1.33 \times 10^{-2}$) |
| DYF326a | 0                | 135     | $0 (0 - 2.70 \times 10^{-5})$ | 6 3239 $1.90 \times 10^{-4}$ ($7.6 \times 10^{-5}$ - $4.0 \times 10^{-4}$) |
| DYF626  | 2                | 135     | $1.48 \times 10^{-3}$ ($1.8 \times 10^{-3}$ - $5.25 \times 10^{-3}$) | 33 3212 $1.03 \times 10^{-2}$ ($7.1 \times 10^{-3}$ - $1.44 \times 10^{-2}$) |
| DYF326b | 0                | 135     | $0 (0 - 2.70 \times 10^{-5})$ | 58 3174 $1.20 \times 10^{-2}$ ($8.50 \times 10^{-3}$ - $1.64 \times 10^{-2}$) |
| DYF627  | 1                | 135     | $7.40 \times 10^{-2}$ ($2.8 \times 10^{-2}$ - $4.08 \times 10^{-2}$) | 45 3289 $1.37 \times 10^{-2}$ ($1.00 \times 10^{-2}$ - $1.83 \times 10^{-2}$) |
| DYF518  | 5                | 135     | $3.70 \times 10^{-2}$ ($1.21 \times 10^{-2}$ - $8.43 \times 10^{-2}$) | 87 3079 $2.18 \times 10^{-2}$ ($1.69 \times 10^{-2}$ - $2.76 \times 10^{-2}$) |
| DYF612  | 1                | 135     | $7.40 \times 10^{-2}$ ($2.8 \times 10^{-2}$ - $4.08 \times 10^{-2}$) | 60 3290 $1.82 \times 10^{-2}$ ($1.30 \times 10^{-2}$ - $2.34 \times 10^{-2}$) |
| DYF449  | 1                | 135     | $7.40 \times 10^{-2}$ ($2.8 \times 10^{-2}$ - $4.08 \times 10^{-2}$) | 35 3140 $1.11 \times 10^{-2}$ ($7.80 \times 10^{-3}$ - $1.55 \times 10^{-2}$) |
| DYF547  | 3                | 135     | $2.22 \times 10^{-2}$ ($4.60 \times 10^{-3}$ - $6.36 \times 10^{-3}$) | 64 3202 $2.00 \times 10^{-2}$ ($1.54 \times 10^{-2}$ - $2.54 \times 10^{-2}$) |
| DYF401S1| 3                | 135     | $2.22 \times 10^{-2}$ ($4.60 \times 10^{-3}$ - $6.36 \times 10^{-3}$) | 41 3262 $1.26 \times 10^{-2}$ ($9.60 \times 10^{-3}$ - $1.70 \times 10^{-2}$) |
| DYF4031a| 2                | 135     | $1.48 \times 10^{-2}$ ($1.8 \times 10^{-3}$ - $5.25 \times 10^{-3}$) | 80 3027 $2.64 \times 10^{-2}$ ($2.10 \times 10^{-2}$ - $3.28 \times 10^{-2}$) |
| DYF4031b| 6                | 135     | $4.44 \times 10^{-3}$ ($1.65 \times 10^{-3}$ - $9.42 \times 10^{-3}$) | 39 2974 $1.31 \times 10^{-2}$ ($9.30 \times 10^{-3}$ - $1.79 \times 10^{-2}$) |
| Across All | 59 | 2025 | $1.60 \times 10^{-2}$ ($2.23 \times 10^{-2}$ - $5.74 \times 10^{-2}$) | 878 47731 $1.84 \times 10^{-2}$ ($1.72 \times 10^{-2}$ - $1.96 \times 10^{-2}$) |

Population genetic analysis in the endogamous Punjabi men

Out of 861 pairs, 75 unrelated Punjabi men, all individuals carried a unique RM Y-STR haplotype with haplotype diversity (HD) 1. In a previous multicenter global study $^{21}$ based on RM Y-STRs where they have reported haplotype diversity of 0.9999985 for almost 12,200 male samples around the globe and for continental regions was ranging from 0.99836 to 0.9999988. In that multicenter global study $^{21}$, several populations also showed the haplotype diversity of 1. We also have compared our current results with previously published populations from Pakistan with Yfiler and RM Y-STRs. On Yfiler 17 Y-STRs, British Pakistani $^{22}$, Punjabi population $^{7}$, Kashmiri population $^{7}$, Hazara Population $^{11}$, Sindh population $^{23}$, Youszai population $^{24}$, and Pathan population$^{25}$ showed DC 99.24%, 87.23%, 68.3%, 76.47%, 86.40%, 71.92%, and 73.7%, respectively. The samples studied with RM Y-STRs showed HD values ranging from 1 to 0.9921 for Pathan $^{21}$, Brahui $^{21}$, Punjabi $^{21}$, Sindhi $^{21}$, Araein $^{26}$, Pakistani $^{6}$, Punjabi$^{19}$ and Sindhi $^{19}$ populations. Multicenter global study $^{21}$ based RM Y-STRs also reported meaningfully lower haplotype diversities and lower unique haplotype proportions in endogamous ethnic groups than in urban and rural groups $^{21}$. Pakistani population is generally considered highly endogamous, and in our previous study 99 pedigrees were sampled from urban (N = 48) and rural (N = 51) areas. We didn't observe any effect on RM Y-STR diversity, which may be due to small sample size. In current study 75 pedigrees were sampled from urban (N = 46) and rural (N = 29) areas and again we didn't observe any effect on RM Y-STR diversity.

### RM marker differentiation per pairs

We have calculated the differentiation power of 13 RM Y STRs in 75 pedigrees. On first meiosis 59 pairs were differentiated out of 135 and DYF399S1 differentiated 24 pairs (40.67%) while DYS526 a/b did not differentiate any pair. This trend was also followed in other pairs where DYF399S1 differentiated most of the pairs while DYS526 a/b did not differentiate any of the pairs (Table 5). This pattern was concordant with previous studies $^{6,15,20,21}$. 

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Informed consent and Ethical approval

The authors declare that they have no conflict of interest.

Conclict of interest

Departmental funds. Zeenat Salahuddin. This study was financially supported by the Department of Forensic Sciences, University of Health Sciences from departmental funds.

Acknowledgments

We thank all volunteers who provided materials and data for this project specially Muhammad Farhan Khan, Saadia Noreen, Midhat Sulman and Zeenat Salahuddin. This study was financially supported by the Department of Forensic Sciences, University of Health Sciences from departmental funds.

Ethics Declarations

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Conflict of interest

The authors declare that they have no conflict of interest.

Informed consent and Ethical approval

Table 5

Male-relative pair combinations differentiation by each of the 13 rapidly mutating Y-STR markers

| Markers          | out of all 135 Father/Son pairs separated | out of all 67 Grandfather/Grandson pairs separated | out of all 104 Brother/Son pairs separated | out of all 147 Uncle/Son pairs separated | out of all 08 Great G. father/G. G. son pairs separated | out of all 190 1st-Cousin/1st-Cousin pairs separated | out of all 14 Grand Nephew/Uncle pairs separated | out of all 168 1st Cousin1r/1st Cousin1r pairs separated | out of all 28 2nd cousin pairs separated |
|------------------|------------------------------------------|--------------------------------------------------|------------------------------------------|------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|------------------------------------------|
| DYS526a/b        | 0(0%)                                    | 0(0%)                                            | 0(0%)                                    | 0(0%)                                    | 0(0%)                                           | 0(0%)                                           | 0(0%)                                           | 0(0%)                                           | 0(0%)                                    |
| DYS612           | 1(0.74%)                                  | 5(7.46)                                         | 5(4.80)                                 | 4(2.72)                                 | 0(0%)                                           | 18(9.47)                                         | 4(0.0%)                                          | 14(8.33)                                         | 4(14.28)                                  |
| DYF399           | 24(17.77%)                                | 12(17.9)                                        | 19(18.2)                                 | 27(18.3)                                 | 1(1%)                                           | 60(31.5)                                         | 4(28.57%)                                        | 48(28.5)                                         | 12(42.85)                                  |
| DYS547           | 3(2.22%)                                  | 2(2.98)                                         | 8(7.69)                                 | 4(2.72)                                 | 0(0%)                                           | 24(12.6)                                         | 2(14.28%)                                        | 19(11.3)                                         | 5(17.85)                                  |
| DYF404           | 3(2.22%)                                  | 4(5.97)                                         | 5(4.80)                                 | 1(0.68)                                 | 0(0%)                                           | 8(4.21)                                         | 0(0%)                                           | 6(3.57)                                           | 2(7.142)                                  |
| DYS626           | 2(1.481%)                                 | 0(0%)                                           | 2(1.92)                                 | 0(0%)                                    | 0(0%)                                           | 0(0%)                                           | 0(0%)                                           | 0(0%)                                           | 0(0%)                                    |
| DYF403           | 8(5.925%)                                 | 7(10.4)                                         | 12(11.5)                                 | 16(10.8)                                 | 0(0%)                                           | 56(29.4)                                         | 0(0%)                                           | 43(25.5)                                         | 13(46.42)                                  |
| DYS756           | 4(2.962%)                                 | 2(2.98)                                         | 3(2.88)                                 | 10(6.80)                                 | 1(1%)                                           | 8(4.21)                                         | 3(21.42%)                                        | 6(3.57)                                         | 2(7.142)                                  |
| DYS518           | 5(3.703%)                                 | 2(2.98)                                         | 2(1.92)                                 | 6(4.08)                                 | 0(0%)                                           | 6(3.15)                                         | 2(14.28%)                                        | 4(2.38)                                         | 2(7.142)                                  |
| DYS627           | 1(0.740%)                                 | 1(1.49)                                         | 1(0.96)                                 | 3(2.04)                                 | 0(0%)                                           | 4(2.10)                                         | 1(7.14%)                                        | 4(2.38)                                         | 1(3.571)                                  |
| DYS570           | 2(1.481%)                                 | 1(1.49)                                         | 1(0.96)                                 | 1(0.68)                                 | 0(0%)                                           | 2(1.05)                                         | 0(0%)                                           | 1(0.59)                                         | 1(3.571)                                  |
| DYS449           | 1(0.740%)                                 | 0(0%)                                           | 1(0.96)                                 | 3(2.04)                                 | 0(0%)                                           | 2(1.05)                                         | 0(0%)                                           | 2(1.19)                                         | 0(0%)                                    |
| DYF387           | 5(3.703%)                                 | 2(2.98)                                         | 1(0.96)                                 | 1(0.68)                                 | 0(0%)                                           | 8(4.21)                                         | 0(0%)                                           | 6(3.57)                                         | 2(7.142)                                  |

Conclusion

63 endogamous pedigrees which contain 327 individuals were analyzed with classic RM Y-STR panel for differentiating male relatives. Usually conventional Y STRs fail to differentiate between paternal relatives from same pedigree. This RM Y STR panel has provided high level of differentiation between paternally related males and across 4th meiosis differentiation rate is >80%. These rapidly mutating Y chromosomal STRs showed the differential ability for both unrelated males and paternal relatives. These markers which are part of RM Y STRs panel also showed high mutation rates. Different commercial companies have proposed different panels of Y Chromosomal STRs to supplement the currently used set of 17 Y-STRs for increased paternal lineage differentiation such as power plex 23 and Yfiler Plus, but none of these showed increase discrimination capacities to differentiated paternal relatives as RM Y STRs have. Moreover, these 13 RM Y STRs give us 29%-100% paternal lineages differentiation in most of the populations and 26%-85% in endogamous populations where conventional Y STRs fails to differentiate or have 4% paternal lineages differentiation power. This high rate of differentiation or individualization using classic RM Y STRs is a great benefit to the field of forensic investigative genetics. Results of this study are concordant with previous studies15,16,20,21 and have showed considerably increased discrimination power. We also merged this data with previous data (and based on 5176 pairs of men related by 1~20 meioses), overall differentiation rate was 38.79% while Y-filer differentiation was 9.24% based on 3864 pairs. Using father-son pair data from the present and previous studies, we also provide updated RM Y-STR mutation rates. However, further studies should be conducted on Pakistani populations, mainly in comparison with commercial kits to further improve the mutation rate information. This study contributes to globally expanding databases for the set of 13 RM Y-STRs.
All participants gave their informed consent either orally (in case they could not write) or in writing after the study aims and procedures were carefully explained to them. The study was approved by the ethical review board of the University of Health Sciences, Lahore, Punjab, Pakistan, and in accordance with the standards of the Declaration of Helsinki 1964.

**Author contribution**

A.R and A. Adnan developed the idea. S.N., M.J. and R.R, collected the samples. S.N., R.A., R.L., A. Alreyami, I.A, and A. Adnan conducted the experiment. A. Adnan., S.N., A.R., and S.H, analyzed the results. A. Adnan wrote the manuscript and A. Adnan and S.H. revised the manuscript. All authors reviewed the manuscript.

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