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ORIGINAL ARTICLE

Analysis of 12 X-STRs in Greenlanders, Danes and Somalis using Argus X-12

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Abstract X-chromosome markers have become a useful set of markers of choice when certain complex kinship cases need to be unravelled. The Argus X-12 kit allows the co-amplification in a single PCR reaction of 12 X-chromosome short tandem repeats located in four linkage groups. A number of 507 unrelated individuals from Greenland, Denmark and Somalia together with two generation families were typed using the Argus X-12 kit. Silent alleles for the DXS10148 and DXS10146 systems were observed in males, mostly from Somalia. High levels of intrapopulation variability and therefore high forensic parameter values were calculated for the three studied populations. The population in Greenland showed a significantly lower intrapopulation variability and a high genetic differentiation compared with 13 other populations. Significant levels of linkage disequilibrium were observed between markers belonging to the same linkage group, mainly in the populations in Greenland and Somalia. Family studies allowed the calculation of mutation and recombination frequencies. A higher male versus female mutation rate was obtained, with an average value of 3.3×10^{-3}. Recombination fraction calculations performed on two generation families showed, as previously described, a not complete independence between X-chromosome linkage groups 3 and 4.

Keywords Argus X-12 · X-STRs · Linkage · Linkage disequilibrium · Greenland · Denmark · Somalia

Introduction

The analysis of complex kinship cases often requires the study of more genetic markers than the commonly used autosomal short tandem repeat (STR) systems to unravel simple cases. X-chromosome markers have become a noteworthy set of markers of choice especially useful for the study of deficiency kinship cases where the father cannot be typed [1]. Polymorphisms of different nature, X-chromosome STRs (X-STRs), X-chromosome single nucleotide polymorphisms and more recently X-chromosome indels have been developed during the last years for use in forensic and population genetics [2-6]. Due to their high polymorphism, STRs are the most widely used genetic markers in forensic genetics. A considerable number of X-STRs and multiplex reactions have been developed by several groups (e.g. [2, 3, 7-10]). A set of 12 X-STRs has recently been combined in a single multiplex reaction and commercialized under the name of Argus X-12 kit [11]. The 12 X-STRs included in the Argus X-12 kit are located in four X-chromosome linkage groups (Xp22, Xq12, Xq26 and Xq28) as previously described [1].

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As it applies to other groups of genetic markers, population frequency databases of X-chromosome markers are needed before its use in forensic investigations. Because all X-chromosome markers are located on the same chromosome, an extra effort is needed in order to investigate haplotype frequencies and the level of linkage disequilibrium (LD) between pairs of loci. In most forensic applications, it will also be necessary to know the genetic stability of the linkage groups and the degree of dependence between them. As discussed by Tillmar et al. [12], considerable different likelihood ratios are often obtained when linkage in families and linkage disequilibrium in populations are not taken into account.

The aim of this study was to analyse the results obtained with the Argus X-12 kit in the Greenlandic, Danish and Somali populations. Allele and haplotype frequencies together with basic population genetic information are provided for each population. Moreover, the analysis of two generation families allowed performing segregation studies. Values of mutation frequencies and recombination fractions were calculated contributing to increase our knowledge of the 12 X-STRs included in the Argus X-12 kit and the stability of the four linkage groups on the X-chromosome.

Materials and methods

DNA samples

Samples from two generation families with one or more children as well as unrelated individuals were collected from Greenland (198 samples), Denmark (210 samples), Somalia (441 samples) and other populations (37 samples). The samples were obtained from paternity and immigration cases where the genetic relationship had been confirmed by the analyses of autosomal markers. The total number of studied duos (woman–boy, woman–girl, man–girl) and the total number of unrelated individuals per population are shown in Table 1. The protocols were approved by the Danish ethical committee (KF-01-037/03).

X-chromosome STR typing

A number of 12 X-chromosome STR markers (DXS10148, DXS10135, DXS8378, DXS7132, DXS10079, DXS10074, DXS10103, HPRTB, DXS10101, DXS10146, DXS10134 and DXS7423) were amplified using the Mentype® Argus X-12 PCR Amplification Kit (Biotype®, AG) and the Investigator Argus X-12 Kit (Qiagen GmbH, Hilden, Germany). The manufacturer's recommendations were followed with the exception that the final PCR reaction volume was 10 μL and the amplification was performed with 25 PCR cycles. A positive control was included in each PCR run. The PCR products were separated by capillary electrophoresis in an ABI3130*xl* Genetic Analyser (Applied Biosystems (AB), Foster City, CA, USA). The electropherograms were analysed using GeneScan® (AB) and Genotyper® 3.7 (AB). Nearly 60% of the samples had previously been typed with Mentype® Argus X-8 kit (Biotype), which included the X-STR loci: DXS10135, DXS8378, DXS7132, DXS10074, HPRTB, DXS10101, DXS10134 and DXS7423. The remaining 40% of the samples were typed two times with Argus X-12 kit.

Sequencing of silent alleles

Silent alleles were observed for the DXS10148 and DXS10146 systems. Most likely, one or more mutations in the primer binding sites reduced the efficiency of the PCR reaction leading to only one allele in females and locus dropout in males. Because we did not know the sequences of the PCR primers used in the Argus-X12 kit, new sets of PCR primers (Supplementary Table 1) were designed up- and downstream to published PCR primers for DXS10148 [9] and DXS10146 [10]. For each system, the same set of primers was used in the PCR and sequencing reactions. Due to the presence of a long poly(A) stretch 3 nucleotides upstream the DXS10148 system, it was only possible to obtain readable sequences for the DXS10148 system by using the reverse primer. The BigDye® Terminator v1.1 Cycle Sequencing Kit (AB) was used to perform the sequencing reactions. Two samples (one from Denmark and one from Somalia) that showed silent alleles for

Table 1 Numbers of samples analysed for 12 X-chromosome STRs with Argus X-12

	Greenland	Denmark	Somalia	Others[a]
Woman–boy duos	31	26	120	16
Woman–girl duos	19	25	112	9
Man–girl duos	19	23	107	6
Unrelated men	98	104	127	4
Unrelated women	50	54	74	8
Total number of individuals	198	210	441	37

[a] The group "others" included individuals from Nigeria, Ghana, Uganda, Iraq, Afghanistan and Myanmar

DXS10148 and two samples (both from Somalia) that showed silent alleles for DXS10146 were sequenced.

Statistical analysis

The Arlequin 3.5 software [13] was used for all population genetic calculations. Allele frequencies were calculated by gene counting in unrelated females and males for each population. Male and female allele frequencies were combined after testing for significant differences in allele frequency distributions. Possible deviations from Hardy–Weinberg expectations (HWE) were tested in females. Haplotype frequencies and LD between pairs of loci were investigated for each linkage group in unrelated men and boys (one boy per family). Observed and expected heterozygosities as well as haplotype diversities were also estimated.

Pairwise F_{ST} values for each X-STR were calculated between a variable number of populations, depending on the X-STR system. Pairwise F_{ST} values based on eight of the 12 studied X-STRs (DXS10135, DXS8378, DXS7132, DXS10074, HPRTB, DXS10101, DXS10134 and DXS7423) calculated for 14 populations in Denmark, Greenland and Somalia (present study), Germany [14], Italy [15], Algeria [16], Finland [17], Somalia [17], Hungary [18, 19], Korea [20], Ghana [21], Poland [22], Japan [23] and China [24] were averaged over loci and represented in a multidimensional scaling (MDS) plot using the statistical package SPSS v.15.0.

Statistical parameters of forensic interest such as power of discrimination in males (PDm) and females (PDf), mean exclusion chance (MEC) in trios involving daughters and MEC in father–daughter duos were calculated using the formulas described by Desmarais et al [25]. Paternity and maternity indices were estimated as likelihood ratios (LR) in father–daughter and mother–son duos from Greenland, Denmark and Somalia. Duos showing genetic inconsistencies (most likely caused by mutations or shared null alleles) were not analysed. Likelihood ratios were obtained using (1) expected haplotype frequencies under linkage equilibrium based on the allele frequencies and (2) the observed haplotype frequencies. A box-and-whisker plot was drawn using SPSS v.15.0 in order to summarize the LR calculated for each population and set of haplotype frequencies used for the calculation.

Female and male mutation frequencies were estimated by combining data collected for the studied populations. Thus, a total number of 358 female meioses and 155 male meiosis were considered for the calculations.

Recombination fractions were calculated from two generation families with two or more children using Mendel v.10 software [26]. The 67 family constellations included in the analysis accounted for a maximum number of 148 informative meioses. Somali allele frequencies were used for the recombination calculations because most of the families used for this analysis were from Somalia. Multiple test P values were corrected using the Bonferroni procedure [27].

Results and discussion

Argus X-8 versus Argus X-12

Discrepant results can be expected between the results obtained using the two kits Argus X-8 and Argus X-12 due to differences in primer design. From the 523 samples analysed with both kits, two discordant results were observed: (1) For the HPRTB system, the allele typed as HPRTB*11.2 with Argus X-8 was typed as allele HPRTB*12 with Argus X-12 in one individual and (2) the allele typed as DXS10134*35.2 with Argus X-8 was typed as allele DXS10134*36 with Argus X-12 in another individual. As it was described for the HPRTB system [28], insertion–deletion polymorphisms up- or downstream to the repeat unit can originate intermediate alleles, depending on the location of the PCR primers.

Silent alleles

Silent alleles on X-chromosome markers can directly be observed in males as locus dropouts. Incomplete X-STR profiles were obtained for some of the male samples, where one of the 12 investigated X-STR systems was missing (DXS10148 or DXS10146). A total number of five male samples showed a locus dropout for the DXS10148 system (one from Denmark, three from Somalia and one from Nigeria). One male sample from Ghana and 16 from Somalia (including 11 boys from four families and five unrelated men) did not show any peak at the DXS10146 system. Two of the samples showing locus dropout at the DXS10148 system and two of the samples missing the DXS10146 system were sequenced (Supplementary Table 2). The two sequences obtained for the DXS10148 system revealed a G-A transition two nucleotides from the 3′ end of the forward primer published by Hundertmark et al. [9]. This mutation, which is most likely the cause of the silent allele found in DXS10148, also changes the internal structure of the repetitive unit (Supplementary Table 2). The two male samples sequenced for the DXS10146 showed the same mutation: a del[CTTT] between 15 and 18 nucleotides from the 3′ end of the reverse primer published by Edelmann et al. [10].

Intrapopulation variability

Allele frequencies and observed and expected heterozygosities calculated for each X-STR and population (Greenland,

Denmark and Somalia) are shown in Supplementary Table 3. A significant lower variability was observed for the population in Greenland compared to the populations in Denmark and Somalia ($P<0.05$, Wilcoxon test). The lowest heterozygosity (0.460) was observed for the DXS8378 system in the Greenlandic population where more than 70% of the sampled alleles were DXS8378*10. The highest variability was observed for DXS10135 system with 33 alleles and an observed heterozygosity of 0.946 in the Somali population. No deviations from HWE were observed with the exception of DXS10148 system in the Somali population ($P<0.05$, after correction). Even though non-significant, a lower observed than expected heterozygosity (0.865 versus 0.939) was also observed for the DXS10146 system in Somali females. The deficit of heterozygotes observed for these two X-STR systems in Somalis can most likely be explained by the presence of silent alleles that when present in females are miscalled as homozygotes.

Haplotype frequencies studied for each of the four linkage groups and for each population (Greenland, Denmark and Somalia) are shown in Supplementary Table 4. The lowest haplotype diversity (HD) was observed in the Greenlandic population. The most frequent haplotype among Greenlanders (DXS7132*14–DXS10079*19–DXS10074*16) was observed 26 times (20%) (Supplementary Table 4). A low percentage of unique haplotypes was observed among Greenlanders (between 15.5% and 19.4%) compared to the percentage observed in the populations in Denmark and Somalia (higher than 32%). The population in Greenland showed the highest level of LD. For this population, significant LD ($P<0.001$) was observed in all loci pairs inside each linkage group. Even though lower levels of LD have generally been observed in African than in European populations [29], this was not the case of the Somali sample, where the level of LD was higher than the one observed for the Danish population. A more exhaustive sampling of both populations (Somali and Danish) would be needed to confirm this observation.

Interpopulation variability

Data for the 12 studied X-STR systems were collected in order to calculate pairwise F_{ST} values between populations (Supplementary Table 5). The highest F_{ST} value was observed for DXS8378 between Greenland and Morocco ($F_{ST}=0.276$, $P<0.0001$; Supplementary Table 5). The X-STR systems DXS10103, DXS10074, DXS8378 and DXS7423 reached F_{ST} values higher than 0.100, which mainly involved the Greenlandic population and pairwise comparisons between Asian and African populations (Supplementary Table 5). Pairwise F_{ST} values calculated between 14 populations for eight of the 12 X-STRs were averaged and represented in a two-dimensional MDS plot (Fig. 1). As shown in Fig. 1, the populations tended to group according to their continent of origin. The clear differentiation of the population in Greenland versus the other 13 populations in the analysis was remarkable. The results are in agreement with previous studies performed on the Greenlandic population. As discussed by Bosch et al. [30], a sex-biased contribution of European genes to the Greenlandic population has probably occurred. Thus, while around 50–60% of the Greenlandic Y-chromosomes seem to have European origin [30-32], a very low European impact was observed from mtDNA data [33]. MtDNA data showed not only a limited intrapopulation variability [34] but also a

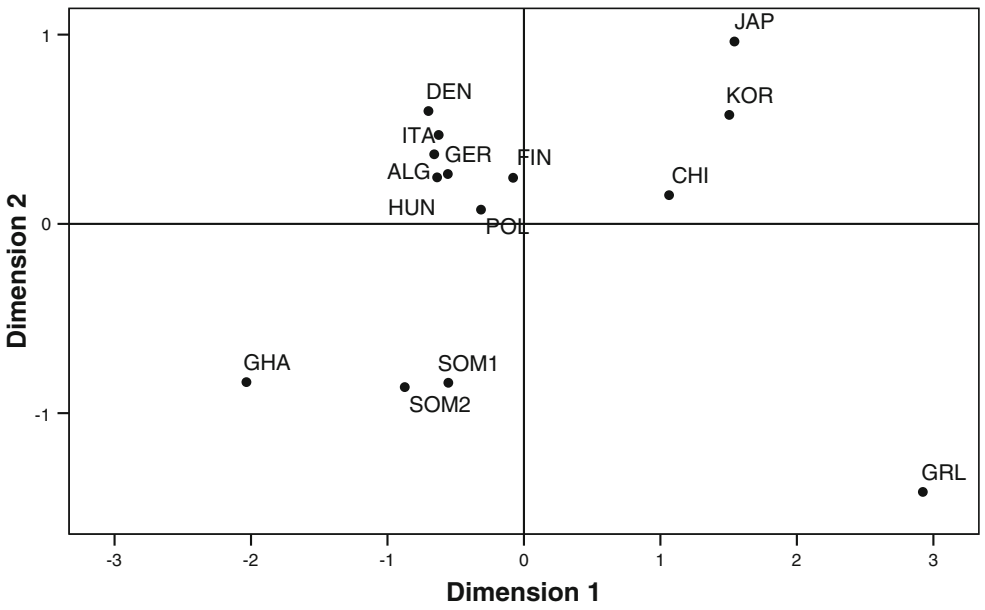

Fig. 1 Two-dimensional MDS plot drawn from pairwise F_{ST} values averaged over eight X-STR loci. *DEN* Denmark, *SOM1* Somalia (this study), *SOM2* Somalia [17], *GRL* Greenland, *GHA* Ghana, *JAP* Japan, *KOR* Korea, *ITA* Italy, *ALG* Algeria, *GER* Germany, *FIN* Finland, *POL* Poland, *HUN* Hungary, *CHI* China

stratification of the population in Greenland [34], which was also evidenced by the analysis of Y-chromosome markers [30, 31]. These results reflect the complex population history of the Greenlanders where both the stochastic effect of genetic drift and interbreeding of different Eskimo cultures have most likely occurred [34]. Nevertheless, the exact origin and interaction between Eskimo groups is still being unravelled [35]. MtDNA data on modern Greenlanders pointed out the possible interbreeding between Neo-Eskimos (Thule) and Paleo-Eskimos (Dorset) [34]. Recent studies based on a single Paleo-Eskimo sample (Saqqaq) suggested an independent origin of Saqqaq and Neo-Eskimo cultures [35, 36]. Because two thirds of the X-chromosomes are from maternal origin, X-chromosome markers will mostly behave as matrilineal markers showing similar results to those obtained from mtDNA data. The observed displaced position of the Greenlandic population based on X-STR results may reflect the differential matrilineal background of this population, probably accentuated by the effect of genetic drift.

Forensic parameters and paternity indices

Forensic parameters of interest, such as PDf and PDm and mean exclusion chances, were calculated for each X-STR and population. The calculations were performed both using allele frequencies (Supplementary Table 3) and observed haplotype frequencies (Supplementary Table 4). The combined PDf calculated from allele frequencies ranged from a value of 1 in 3×10^{12} (in Greenlanders) to 1 in 1×10^{17} (in Somalis). Lower values were obtained when haplotype frequencies were taken into account (from 1 in 3×10^{9} to 1 in 2×10^{14}). Combined PDm values ranged from 1 in 2×10^{5} (calculated from haplotype frequencies from Greenland) to 1 in 8×10^{9} (obtained from allele frequencies from Somalia). Also a high combined MEC was obtained for father–daughter duos and father–girl–mother trios (>0.9999 in all cases).

Paternity and maternity indices were calculated as LR for father–daughter and mother–son duos from Greenland, Denmark and Somalia using allele and haplotype frequencies (Fig. 2). As expected from the values of intrapopulation diversity, the lowest median LR values were observed for the Greenlandic duos. LR values calculated from allele frequencies (expected haplotype frequencies under linkage equilibrium) were between one and two orders of magnitude higher than those calculated from observed haplotype frequencies. A limited sample size and the existence of linkage disequilibrium inside linkage groups can explain the differences in the calculated LR values, as previously discussed by others [12, 15]. Linkage disequilibrium inside linkage groups requires the use of observed haplotype frequencies instead of its expected value [12]. Due to the low frequency observed for each haplotype, large population databases are required in order to obtain more accurate LR values.

Segregation analyses

A total number of 20 mutations were observed in a cumulative number of 6,156 meiosis for the 12 X-STRs (average mutation rate=3.3×10^{-3}; Table 2). This value fell

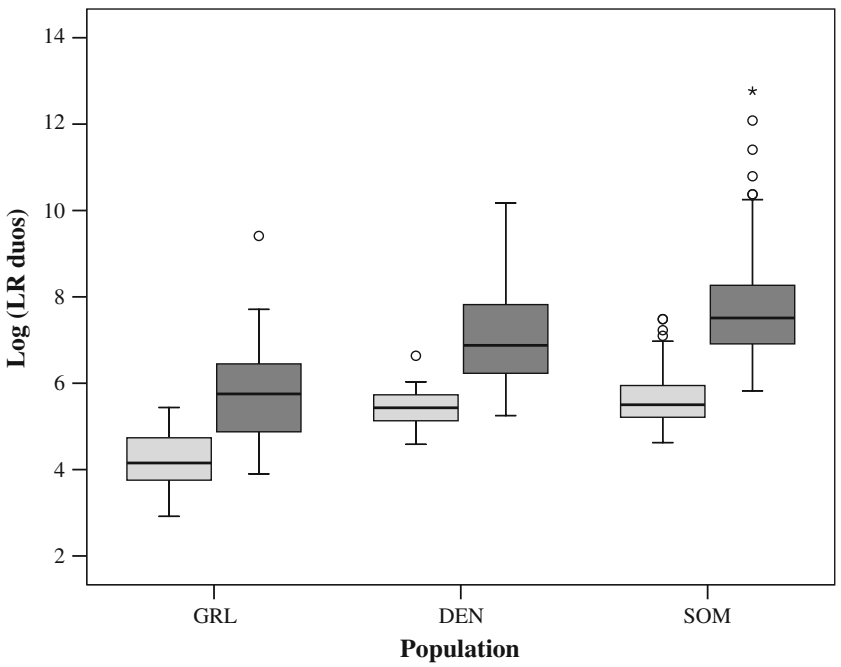

Fig. 2 Box-and-whiskers plot of LR values calculated from allele (*dark grey*) and haplotype frequencies (*light grey*) in Greenlanders (*GRL*; data based on 49 duos), Danes (*DEN*; based on 43 duos) and Somalis (*SOM*; based on 187 duos). The *boxes* represent the first, the median and the third quartile, and the *whiskers* show the minimum and maximum values. The cases with values that are between 1.5 and 3 box lengths from either end of the box are represented by *circles*, and the cases with values more than 3 box lengths from either end of the box are represented by *asterisks*

Table 2 Mutation frequencies estimated for 12 X-STRs

System	Females (358 meiosis)		Males (155 meiosis)		Total (513 meiosis)		95% CI[b] ($\times10^{-3}$)
	N	($\times10^{-3}$)	*N*	($\times10^{-3}$)	*N*	($\times10^{-3}$)	
DXS10148	0	<2.8	2	12.9	2	3.9	0.5–14.1
DXS10135	2	5.6	3	19.4	5	9.8	3.2–22.7
DXS8378	0	<2.8	0	<6.5	0	<2	0–7.2
DXS7132	1	2.8	0	<6.5	1	2	0.05–10.9
DXS10079	1	2.8	3	19.4	4	7.8	2.1–20
DXS10074	0	<2.8	1	6.5	1	2	0.05–10.9
DXS10103	1	2.8	1	6.5	2	3.9	0.5–14.1
HPRTB	0	<2.8	0	<6.5	0	<2	0–7.2
DXS10101	0	<2.8	0	<6.5	0	<2	0–7.2
DXS10146	0	<2.8	2	12.9	2	3.9	0.5–14.1
DXS10134	1	2.8	1	6.5	3[a]	5.9	1.2–17.1
DXS7423	0	<2.8	0	<6.5	0	<2	0–7.2
Cumulative	6	1.4	13	7	20	3.3	2–5

[a] Includes a female or male mutation

[b] 95% Poisson confidence interval

inside the 95% confidence interval (1.3×10^{-3}–3.3×10^{-3}) calculated for 16 X-STRs by Szibor et al. [1]. Mutation rates of the same order were also described for autosomal STRs [37]. A higher male versus female mutation rate was observed (Table 2). This sex difference can also be observed for autosomal STRs (http://www.cstl.nist.gov/strbase/mutation.htm).

The silent alleles described for DXS10148 and DXS10146 could indirectly be observed in females from families where parents and children did apparently not share any allele for those X-STR systems (e.g. mother–girl "opposite homozygosity"). Silent alleles for DXS10146 were presumably observed in 14 females while silent alleles for DXS10148 were concluded in 13 female samples.

An unbalanced tri-allelic pattern was observed for the DXS10146 system in two unrelated women. In one of the cases, the peak height ratio between the three peaks was approximately 3:1:3, and the lowest peak was one repeat unit smaller than one of the main alleles. In the other case, the peak height ratio was around 4:2:1, and the lowest peak was one repeat unit longer than the second lowest allele. In the first case, the parents and the sister of the girl showed a normal allelic pattern. The minor allele present in the girl was not observed in any of the studied relatives. In the second case, two sons and two daughters of the woman were investigated. All of them showed a normal pattern, and none of them had the minor allele present in the mother. Usual amounts of DNA were used in the PCR reactions, and large stutters were not expected. The observed unbalanced tri-allelic patterns could be explained as the result of a somatic mutation as described by Rolf et al. [38]. Similar aberrant signals were observed for DXS10079 and DXS10074 [8].

Recombination frequencies

Recombination fractions were calculated from two generation families with two or more children (most of them from

Table 3 Recombination fractions calculated for the 12 X-STRs included in Argus X-12

Marker 1	Marker 2	Maximum LOD score	Rf	Significant meiosis[a]
DXS10148	DXS10135	41	0.0001	137
DXS10135	DXS8378	33	0.0001	109
DXS8378	DXS7132	0	0.5000	92
DXS7132	DXS10079	27	0.0132	108
DXS10079	DXS10074	38	0.0001	133
DXS10074	DXS10103	0	0.5000	116
DXS10103	HPRTB	34	0.0001	113
HPRTB	DXS10101	38	0.0001	129
DXS10101	DXS10146	1	0.3142	134
DXS10146	DXS10134	42	0.0001	140
DXS10134	DXS7423	25	0.0078	93

Rf recombination fractions

[a] Maximum number of meiosis = 148

Somalia). A number of 67 family constellations were studied which accounted for a maximum number of 148 significant meiosis. Table 3 shows the results obtained from the recombination study. Between 92 and 140 significant meiosis were observed in the 11 pairs of markers. The highest stability was observed in linkage groups 1 and 3. Although recombination fractions obtained from two generation families are not as accurate as those obtained from three generation families, the not complete genetic independence (<50 cM) obtained between linkage groups 3 and 4 is in accordance with previous studies [1, 15, 39].

Conclusions

A total number of 886 samples were analysed for 12 X-STR using the Argus X-12 kit. Around 60% of the samples were also analysed with Argus X-8 kit, and few discrepancies were observed between the results obtained from both kits. Two of the new X-STR systems introduced in Argus X-12 (DXS10148 and DXS10146) showed silent alleles, mostly observed in the Somali population. The results obtained in the Somali population suggest that the application of the Argus X-12 kit in non-European populations may require the re-design of PCR primers for the DXS10148 and DXS10146 systems.

As described for other populations, a high level of polymorphism was observed for the 12 genetic markers included in Argus X-12 kit in the three populations analysed in this study. A significant lower variability together with a high genetic differentiation from the other investigated populations was shown in the population in Greenland. Significant linkage disequilibrium was observed between markers inside linkage groups, especially in Greenlanders. As previously discussed by others, haplotype frequencies of each linkage group instead of allele frequencies need to be used in forensic cases. In order to increase the forensic utility of the combined X-STR markers, larger databases are then required.

Higher male than female mutation frequencies were observed. An average mutation rate of 3.3×10^{-3} was obtained by combining data from the studied populations. This value is in agreement with previous studies. Recombination fractions calculated from two generation families showed, as described by others, that linkage groups 3 and 4 are not completely independent.

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Conflict of interest The authors declare that they have no conflicts of interest.

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