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Review Article

Applications of X-Chromosome Short Tandem Repeats for Human Identification: A Review

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ABSTRACT

The forensic DNA profiling technique has tremendously contributed to forensic human identification, an important aspect in forensic investigations. In instances whereby comparison samples are unavailable, utilization of short tandem repeats of X chromosome (X-STRs) may prove useful to resolve complex kinship investigations involving missing persons and mass disasters. Despite such evidential values, the use of X-STRs during investigations remains scarce in many Southeast Asian countries including Malaysia, requiring concerted efforts for establishing forensic statistical support for its diverse populations (especially the admixture populations), standardizing core loci and procedure, improving the knowledge among practitioners as well as developing suitable standard operating procedure for incorporating X-STRs analysis in the overall DNA profiling framework. Hence, this review paper aims to highlight the developments, applications and population data of X-STRs, as well as its challenges and future insights for forensic casework.

Keywords: Complex kinship, Forensic statistical parameters, Human identification, Population genetics, Short tandem repeats, X-chromosome

Introduction

X-STR profiling and its evidential values

Forensic DNA profiling is associative evidence linking the biological evidence found at the crime scene with a reference DNA sample (suspect or victim) for the identification or exclusionary purposes [1]. This technique has gained recognition as a powerful tool in human identification, especially in the criminal justice community, since its first introduction and popularization by Sir Alec Jeffreys in 1984 [2]. The first case reported on the utilization of DNA profiling was in resolving a deficiency paternity testing of an immigration dispute in the United Kingdom [3]. However, it was Colin Pitchfork's case involving the investigation of a double murder of two young girls, where DNA profiling was first utilized for forensic investigation [4]. As such, DNA profiling appears useful in identifying biological evidence [5], and attempting to complete the forensic linkage triangle [6]. A crime can happen even with minimal

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physical contact between the (a) perpetrator and a victim, (b) the victim to the crime scene and (c) the perpetrator to the crime scene [6]. In such a situation, the evidence transfer can be minute (e.g. hair falls or skin shed naturally), and hence, the invention of Polymerase Chain Reaction (PCR) that markedly improves the sensitivity of DNA analysis proves pertinent in enabling successful DNA profiling interpretation [7].

It has been indicated that the human genome contains many repetitive DNA sequences (microsatellites), i.e. repeated in variable numbers among different individuals that can be informative for discrimination of identities [8], a significant aspect in forensic investigation. In addition, DNA profiling has also been utilized to resolve paternity and kinship issues, development of DNA database search, and forensic intelligence [1, 9]. In this context, the utilization of autosomal Short Tandem Repeats (STRs) has acquired considerable attention due to its evidential value in establishing individualities [10]. Unfortunately, the interpretation of DNA profiles derived from autosomal STRs requires the existence of suitable comparison samples, which may not always be available in every case. In the absence of comparison samples, the generated DNA profiles may not provide meaningful identification of individuals [11, 12]. In particular, forensic scientists have to face difficulties in complex kinship investigations cases (a) where the relatedness among individuals are in-questioned and (b) the parents are missing as well as (c) involving complex pedigree [13, 14]. These conditions would unwantedly result in inconclusive likelihood ratio (LR) support [14].

While an autosomal STR refers to a marker on 'a chromosome not involved in sex determination', an X-chromosomal STR (X-STR) is 'the marker found on the X chromosome that can sometimes be useful in addressing kinship analysis' [9]. The utilization of X-STRs DNA profiling is deemed important in complementing the autosomal STRs DNA profiling, particularly in complex kinship cases (e.g. missing persons and mass disasters) [15, 16], whereby inconclusive or less informative autosomal STRs results are obtained [17]. This is because the STRs markers located on the X-chromosome contain genetic information from both the genders [18] with unique mode of inheritance [13]. In contrast, the Y-chromosome and mitochondrial DNA (mtDNA) [19] only contain genetic information from the male and the female lineage, respectively. Acknowledging the potential applications of X-STRs markers in forensic investigations, previous researchers provided a summary that included (a) paternity testing in trios and duos, (b) complex kinship testing inclusive of deficiency paternity, (c) incestuous cases relating close blood-relatives and (d) maternity testing [13, 20]. The same authors further indicated that the X-STRs analysis may identify female DNA in the mixture of both the male and female exhibits (e.g. mixed semen stain and nail scrapings) more efficiently than that of autosomal STRs, considering that 'female alleles can only be completely included in the male component if the female coincidentally happens to be homozygous at all loci'. Moreover, the X-STRs analysis can potentially provide the information on genetic anomalies such as Klinefelter syndrome [21] and Ullrich-Turner syndrome [13] that can be used as forensic intelligence for narrowing down the search for individuals in the absence of comparison DNA profiles. However, the application of such an information on genetic anomalies has triggered substantial ethical debates with regards to the appropriateness of unveiling ones' health condition[18]. Having considered the benefits and disadvantages discussed, the assumption of bona fide prevails over the practice of forensic science in unveiling the truth through scientific analysis like DNA profiling for ensuring justice to the victims.

The scientific technique utilized in forensic DNA profiling shall be tested for its validity, potential error and acceptance criteria based on the principle of admissibility of evidence such as that of Daubert standards [4], including X-STRs DNA profiling. As such, the weight of the evidence must be taken into account since the markers used are located on the single X chromosome alone that may affect the biostatistical evaluations [19]. Haddish et al. [22] further accentuated that genetic information (e.g. allele and haplotype frequency) from relevant population is essential to weigh the evidence utilized in forensic identification, particularly in kinship testing for ensuring the correct representativeness of the genetic allele variation [19] of the X-STRs markers used. Therefore, performing the forensic statistical parameters evaluation for the different populations would provide the empirical support for the use of DNA evidence for human identification in the court of law. Need-

less to say that aspects pertaining to the understanding of X-STRs analysis among the legal practitioners, forensic scientists and investigators must be enhanced, apart from providing a conducive environment for incorporating X-STRs analysis in the routine DNA profiling framework.

Rationale of this review

The practical value of analyzing X-STRs for forensic applications has been acquiring popularity in the body of literature, particularly due to its usefulness in complex kinship investigations, as well as in situations whereby comparison samples are missing for the purpose of human identification. While review of literature gives a specific emphasis on the variations observed in the forensic statistical supports for the different monoethnic populations (pure lineages), the same for the different admixture populations that are prevailingly observed worldwide remains scarce, limiting the scientific understanding on the application of X-STRs analysis for cases involving admixtures. In addition, specific attempts to address the current state of knowledge and understanding about the application of X-STRs analysis and its suitability among forensic investigators and legal personnel remain unreported in the literature. While the incorporation of X-STRs analysis in the overall DNA profiling framework is integral for forensic investigation, it is apparent that the existence of such framework is limited to a few countries alone with no available information pertaining to the systems in Asian countries like Malaysia as well as other developing and under developing countries. Considering all these facts along with the other important scientific information about X-STRs, this review that emphasizes to discuss issues relating to admixtures, knowledge and understanding among forensic investigators and legal personnel, as well as the available frameworks and their suitability merits scientific and forensic considerations.

Main Body

X chromosome properties and mode of inheritance

Studies on the X chromosome properties have been documented in the body of literature [23, 24], focusing on its genomic content. In an attempt to represent the human genome, Bentley et al. [23] constructed maps for eight chromosomes (1, 6, 9, 10, 13, 20, X and 22) using building landmark maps, isolating bacterial clones and assembling contigs. The authors described that the estimated length of the X chromosome (p21- q27) being about 115 million base pairs (Mb). The estimated length of the X chromosome was later corrected by Ross et al.[24] as 155 Mb, and this was done by integrating their newly constructed map of the X chromosome (using P1-artificial chromosome (PAC) and bacterial artificial chromosome (BAC) clones) with those reported by Bentley et al. [23]. While X chromosome is one of the biggest chromosomes in human cell [25], it contains high number of interspersed repeats (56%) with low amount of guanine and cytosine content (39%). This situation makes the X chromosome as the gene-poor ones with only 1.7% of its sequence represents the exons that codes for protein. Despite having a low gene density and length, the X chromosome houses the largest gene in human genome named dystrophin [24]. The gene is responsible for maintaining the structural integrity of muscle fibers as well as neuromuscular synapse stability [26].

In human, the two sex chromosomes (X and Y) are responsible in determining the sex of an individual and they are genetically and morphologically different [27]. Researchers have also demonstrated that the X chromosome contains over 800 genes, which are not only responsible for sex determination, but also for several somatic characteristics (e.g. neuronal development) [28]. Between the two chromosomes, the utilization of X-chromosome has gained considerable attention in forensic investigation [18], attributable to its special genetic mode of inheritance [19] as a tool of choice in kinship analysis [29]. Female has two X chromosomes which are similar in size and genetic content [27] and each of them is transmitted one from the father and mother, respectively [29, 30]. Gomes and Arroyo-Pardo [31] accentuated that the pair of X chromosomes in a female behaves like the autosomes, whereby they directly recombine with each other during meiosis, and therefore, the mother randomly transmits a recombined X chromosome copy to her daughters and sons. In contrast, a male inherited one X chromosome from the mother and the Y chromosome from the father [30].

Interestingly, a male has X and Y chromosomes that are non-homologous [28], except for two limited regions of identical sequence on both chromosomes called the pseudoautosomal regions 1 and 2 (PAR 1 and PAR 2) [27, 32]. While PAR

1 (2.6 Mb) is located at the short-arm tips of both the X and Y chromosomes, the PAR 2 (320 kb) can be found at the tips of the long arms (both chromosomes) [32] (Figure 1). During meiosis in males, the pairing and cross over occur only at PAR 1 and PAR 2 regions, whereas in females the same processes transpire throughout the entire length of the X chromosome [27]. With regards to the mode of inheritance, genes that are located at the PAR regions will be in inherited in an autosomal pattern instead of based on sex-linked mode of inheritance and recombined during meiosis [32]. Considering that the X chromosome is transmitted (unchanged) by the father to all his daughters [14], sisters and half-sisters fathered by the same man would have the exact paternal X chromosome (except for mutation) [25, 29]. This fact is particularly useful in paternal half-sisters and paternal grandmother or granddaughter relationship investigations [25] as well as explaining clinical genetic diseases [13]. The former premise of argument is further supported by the statistical inferences made by Pinto et al. [33], whereby the use of theoretical identity-by-descent (IBD) framework of X chromosome mode of transmission would provide better resolution for interpreting individualities.

It has to be acknowledged that there is an imbalance proportion of sex chromosomes and its gene content between the male and female [28]. Lyon [34] reported about the X chromosome inactivation mechanism that occurs in the development of every somatic cell of females, whereby the females eventually have one functional copy of X chromosome to compensate the differences [28]. It has been reported that, X chromosome has lower genetic diversity when compared to that of autosome, although the genetic diversity remains higher than that of the Y chromosome. Moreover, the genetic diversity of X chromosome appears lower in males when compared to with that of females, attributable to lack of second copy of the X chromosome in males [35]. Taking into account the potential use of X-STRs analysis, discussion on the development of varying multiplex X-STRs markers, X chromosome insertion-deletion (X-INDELs) and X chromosome single-nucleotide polymorphisms (X-SNPs) for complex kinship investigations is provided in the next section.

Development of X-STRs analysis

Vergani [8] defined a genetic marker as a "DNA sequence with known physical locations on chromosomes". The STRs are located in the non-

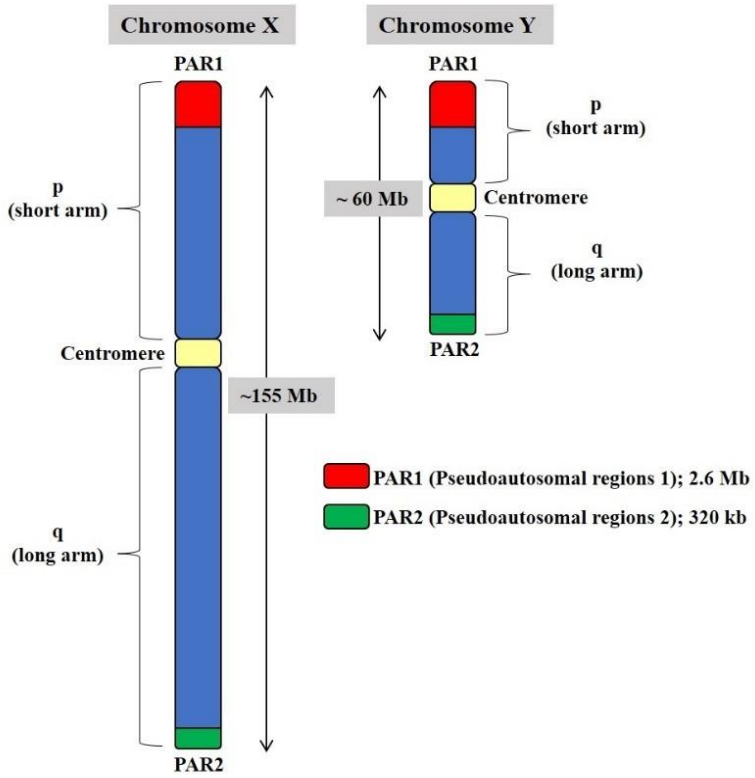

Figure 1. The location of PAR regions on both X and Y chromosomes.

coding regions and contain repetitive DNA sequences, and based on the number of repeats, they are categorically referred as minisatellites (8-100 bp) and microsatellites (2-7 bp) [8]. Owing to its high degree of sequence variations among individuals and populations [25], they are effective markers for human individualization [2]. In view of such an application, Vergani [8] emphasized on the importance of selecting DNA markers that are highly informative and discriminative for individualization, considering the ability of the PCR technique to simultaneously amplify multiple target DNA sequences in the form of STR multiplexes.

Lately, the utilization of genetic markers located on X-chromosome in forensic human identification has been seen as an important tool for forensic investigation, particularly in resolving complex kinship cases and for addressing the limitations associated with autosomal STRs [36]. Historically, it was reported that the early discovery of X-STRs was in the field of clinical genetics in which many diseases and traits were associated with X-chromosome mode of inheritance [13]. The fact that there are few genetic markers useful for forensic application, X-STRs analysis is the most preferred ones, as compared to X-INDELs and X-SNPs [31]. The preference for X-STRs analysis is due to (a) its high polymorphism, (b) easy in genotyping using PCR and (c) the possible use of multiplex amplicons which may prove useful in degraded specimens often recovered during crime scene investigation. As opposed to X-STRs, X-INDELs and X-SNPs analyses suffer from low polymorphism (requiring the involvement of a much higher number of INDELs loci) and the lack of commercial kits, as well as the lack of standardization of methodologies for typing (inter-laboratorial comparisons), respectively [18].

The development in the popularity of X-STRs over the last two decades has contributed to the abundance of X chromosome markers and multiplexes for population genetics and forensic investigations. Szibor et al. [20] revealed that a total number of 26 trinucleotide and 90 tetranucleotide polymorphism on X chromosome are available, and the authors further reviewed 17 of X-STRs markers that are commonly used in forensic casework. The 17 X-STRs listed by the authors are from the work on 16 X-STRs loci frequency data from a German population performed by Edelmann et al. [37] and one locus (DXS11001) by Watanabe et al. [38]. In this context, Szibor et al. [20] concluded that the information on precise location of more X chromosome markers along with quantification of linkage disequilibrium would require concerted efforts for forensic applications.

In a later development, Gusmão et al. [39] evaluated the performance of X-STRs Decaplex that was widely used by the Europeans researchers. The X-STRs Decaplex (DXS8378, DXS9898, DXS7133, GATA31E08, GATA172D05, DXS7423, DXS6809, DXS7132, DXS9902 and DXS6789) was developed by the Spanish and Portuguese Working Group of the International Society for Forensic Genetics (*GEP-ISFG*) as a collaborative exercise among participating laboratories. Despite the fact that the Decaplex appears technically robust, the lack of population and mutation data for forensic evaluation of the multiplex may hinder its real practical values, necessitating scientific endeavours in this aspect.

In this regard, Diegoli [14] first reported about 39 different X-STRs markers used in various multiplexes for population studies as well as kinship investigations, and as reported by Gomes et al. [18], the list has increased tremendously to 85 X-STRs markers, indicating its substantial interest in the literature. However, the Qiagen Investigator® Argus X-12 QS (quality sensor) that is made of 12 X-STRs markers, appears as the most widely used commercial kit available for forensic application.

The first commercialized X-STRs kit was Mentype® Argus X-UL PCR amplification kit (BioType AG) that co-amplified four X-STRs markers namely DXS8378, HPRTB, DXS7423 and DXS7132 plus Amelogenin in a single dye channel [40, 41]. Subsequently, other four X-STRs (DXS10134, DXS10074, DXS10101, and DXS10135) were included into the improved Mentype® Argus X-8 kit [42]. Further, Scherer et al. [43] developed and validated the Qiagen Investigator® Argus X-12 QS kit, an expansion from those earlier kits produced by Biotype AG, by adding four more markers (DXS10103, DXS10079, DXS10146 and DXS10148) in the four dye channels. Not only that Qiagen Investigator® Argus X-12 QS kit has increased number of loci, the PCR cycling protocol was shortened and now could tolerate the presence of high inhibitor with the usage of Fast Reaction Mix 2.0. In addition, the insertion of (a) QS (as internal control) to monitor PCR performance, (b) SNP primers to resolve primer binding site mutations at DXS10101, DXS10146 and DXS10148 and (c) autosomal

marker D21S11 to minimize potential risk of switching samples have significantly increased the robustness of the assay, resulting in it being the most utilized commercial kit for forensic application worldwide.

Several other commercial multiplexes have also been described in the literature [44–46] with most of them are from China. Led by AGCU ScienTch Incorporation (Wuxi, China), Yang et al. [46] developed and improvised the 19 X-STRs multiplex (AGCU X19 STR Kit) from the previous AGCU X12 STR system [47]. Among others, the newly developed kit included 11 loci that were previously used in the Qiagen Investigator® Argus X-12 QS Kit. Next, the Goldeneye17X system (another multiplex developed by Peoplespot, China) was reportedly using 5 colour fluorescent labelling technology that had enabled the amplification of 16 X-STRs loci and a sex determining locus (Amelogenin) in a single reaction [45]. The same authors further assessed genetic polymorphism of the multiplex on the Han population of Shandong province, China. While the kit utilized 16 X-STRs loci, the results of the analysis on the females of Han population revealed that 6 of the 16 loci (DXS9902, DXS8378, GATA165B12, DXS6800, DXS6807 and DXS6810) had relatively low polymorphic information content (PIC) (<0.5), suggesting that the loci may not be discriminative for forensic applications. Currently, a commercial kit (Microreader™19X ID System kit) that comprises of 19 X-STRs has been developed by Microread Genetics Co (Suzhou, China) [44] although its utilization in genetic population studies remains limited to one study on Han and another for Sierra Leone populations. As such, it can be seen that, AGCU X19 STR and Goldeneye17X kits are the preferred commercial kits for X-STRs analysis for forensic applications.

Apart from using the commercially available kits for X-STRs analysis (e.g. Qiagen Investigator® Argus X-12 QS), utilization of in-house multiplexes for X-STRs profiling has also been reported. For instance, Nakamura et al. [48] improvised their previously reported 15 X-STRs multiplexes [49] to an 18 X-STRs multiplex within a single PCR reaction, by adding three additional loci (DXS9902, DXS6795 and DXS6810). In another example, 17 X-STRs loci were utilized by Prieto-Fernández et al. [50], with 10 of which were similar to Decaplex GHEP-ISFG, combined with additional 6 other loci (DXS6801, DXS6799, DXS6800, DXS10075, DXS6807, and DXS6803) and one specifically from the Qiagen Investigator® Argus X-12 QS kit. Progressively, evaluation of several other novel X-STRs markers at specific regions (Xp21.1, Xq21.31, Xp22.32, Xq23 and Xp11.4) for resolving complex kinship cases [51–53] have also been reported. However, when tested on Japanese population, low PIC (ranging from 0.5606 to 0.7448) was obtained for the two regions analyzed (Xp22.3 and Xp11.4) [51–53], indicating that the markers are not well polymorphic.

In a case involving two sisters with paternal dispute, the use of 12 X-STRs could not determine the actual biological father since the two alleged fathers were cousins (with their mothers being sisters) [54]. Considering such a failure in resolving the paternity dispute, Hering et al. [55] later suggested the expansion of the German population data by adding two clusters of X-STRs from previous researchers [51, 56] in their newly designed hexaplex PCR that was combined with the existing Qiagen Investigator® Argus X-12 QS kit. The fact that the increasing number of X-STRs loci used in multiplexes would proportionately increase the power of discrimination among individuals, attempts by researchers to combine several multiplexes (> 20 X-STRs loci) described previously for reporting the relevant population data [57–59] may prove relevant. However, this type of work can be tedious and time consuming since it requires different amplification for the different types of multiplex or single-plex. This problem has caused the lack of practicality for forensic laboratories to adopt this at a large-scale application. Nonetheless, the discovery of novel X-STRs markers should be encouraged among researchers to further investigate the more polymorphic loci for forensic applications; however, without the suitable standardization of X-STRs core loci, the forensic applicability of the markers cannot be positively distinguished. Not only that, the utilization of various multiplexes other than the commercial ones may result in discrepancies of laboratory interpretation with regards to X-STRs data.

The fact that forensic specimens are often degraded [60], utilization of mini STRs of the X chromosome may provide a better result than that of multiplexes; however, limited studies [61–64] are reported in this context. Mini STRs refer to the utilization of small target regions (amplicons,

<200 bp) for forensic identification [65]. In an attempt to mimic a real forensic situation whereby the DNA specimen can be degraded, Asamura et al. [61] introduced two mini-X-STRs multiplex systems that consist of eight X-STRs loci (DXS7423, DXS6789, DXS101, GATA31E08, DXS8378, DXS7133, DXS7424, and GATA165B12). The authors reported that while the amplicons of the PCR primers were made as short as 76 – 169 bp, 22 out of 29 degraded samples analyzed were successfully profiled for at least four loci, suggesting potentially positive identification. Despite the positive results reported by the authors, it is imperative to mention that the evaluation was only made on male samples (haploid) to avoid genotyping error due to allelic drop-out [61]. As such, the fact the female samples were not evaluated, its practical values for representing females (heterozygotes) in degraded specimens remain untested.

Next, Diegoli and Coble [64] optimized two mini-X-STRs multiplexes (*viz*. 8-plex and 10-plex) from the potential small amplicon X-STRs markers based on the heterozygosity and linkage groups across the X chromosome reported earlier. Considering the overlapping X-STRs markers between the two mini multiplexes, the total number of X-STRs markers investigated were 15 *viz.* DXS6789, DXS7130, DXS9902, GATA31E08, DXS7424, GATA165B12, DXS101, DXS6795, GATA172D05, DXS10147, DXS8378, DXS7132, DXS6803, HPRTB and DXS7423. To demonstrate the sensitivity of the two mini-X-STRs multiplexes on degraded DNA samples and the imbalance of amplification efficiency between the two multiplexes in DNA recovery, the authors utilized aged bone specimens (>65 years old) in their analysis. While reporting high degrees of polymorphism and sensitivity (200 pg of DNA), as well as suggesting the potential use of the two multiplexes for forensic analysis of compromised bone samples, the suitable comparison of the results with those of similar larger amplicon multiplexes was not performed. As such, further concordance studies may be necessary. Peculiarly, while justifying the development of seven new mini-X-STRs for analyzing degraded DNA samples, Israr et al. [63] utilized only fresh blood samples from the Punjabi population in Pakistan. The X-STRs investigated were DXS101, DXS6789, DXS7132, DXS7423, DXS8378, GATA172D05 and GATA31E08, and the authors reported a total of 51 alleles (5 – 10 alleles for each marker). The fact that no degraded sample was used, the practical value of the mini-X-STRs developed by the authors remains uncertain.

In addition to X-STRs and mini-X-STRs multiplexes, the use of X-SNPs and X-INDELs for human identification in complex kinship investigation has also been reported in the literature [31]. Due to high occurrence of single base variations (substitutions, insertions or deletions) in human genome, SNPs markers can be considered for individualization [8]. Pertinently, Gill [66] indicated that the minimum of 50 loci are required for SNPs analysis for producing an acceptable probability of paternity exclusion (given the genotype of the mother) that is comparable to the existing STRs multiplexes. Despite such an indication, review of literature still reveals subsequent studies on X-SNPs analysis that utilized lesser number of loci *viz*. 5, 10, 14, 16 and 25 loci, respectively [18]. Therefore, the interpretation of individualities following the use of lesser number of loci than 50 for X-SNPs analysis can be subjected to significant dispute whenever the evidence is presented in the court of law, in violation of the Daubert standard of admissibility of forensic evidence. In view of such a legal matter, it can be seen that newer researchers attempted to address the limitation in the body of knowledge using two different approaches. The first approach is by increasing the number of loci used in X-SNPs analysis, while the second being the incorporation of the lesser number of loci with other prevailing DNA markers [18]. For example, Li et al. [67] utilized 52 X-SNPs in four different multiplexes which may appear to cause difficulties in genotyping process. To overcome such a problem, Stepanov et al. [68] analyzed the genetic diversity of 62 X-SNPs in only two multiplexes among four North Eurasian populations representing Siberia (Buryat and Khakas), North Asia (Khanty) and Central Asia (Kazakh). As for the second approach, Hwa et al. [69] utilized the combination of 220 SNPs (17 X-chromosomal, 30 Y-chromosomal, 48 mitochondrial and 125 autosomal) among Caucasian, East and Southeast Asian populations for the purpose of differentiating the ethnic of origin. Their results revealed the overall accuracy rate of ancestry inference that ranged between 70.0% and 94.4%, depending on the ethnicity investigated, which may provide useful investigative leads in identification of suspects. Although promising results have been

reported by previous researchers with regards to the use of X-SNPs analysis for forensic identification of individuals, Gomes et al. [18] cautioned about the complexity of the data interpretation in cases involving multiple-donor samples that can be observed in contaminated DNA specimens as well as in mixtures. Therefore, concerted effort to resolve this imminent issue proves as forensically necessary.

INDELs (diallelic markers) on the other hand are length polymorphism based on the insertion or deletion of one or more nucleotides in the genome that derived from a single mutation event [8]. Zhang et al. [16] described the advantages of INDELs analysis as (a) having low mutational rates, (b) suitable for degraded DNA due to short amplicon size and (c) easily detected on CE platform; signifying the practical the utilization of INDELs in human identification, population genetics and biogeographic research. Besides, INDELs have the combined features of both STRs and SNPs [70], and therefore, similar approach for combining the markers in the INDELs system has been reported [71, 72]. This is to increase the number of INDELs loci, demonstrating its efficiency in discriminating individuals. For example, Tao et al. [72] attempted to design the SifaInDel 45-plex system which can simultaneously amplify 45 different INDELs markers (27 autosomal, 16 X and 2 Y chromosome INDELs) in a single PCR reaction followed by the genotyping on the CE platform. Based on the forensic statistical parameters computed by the authors, high combined power of discrimination (PD) in male (0.999845) and female (0.999998) as well as mean exclusion chance (MEC) in duos (0.976220) and trios (0.998163) were obtained. The fact that relatively strong forensic statistical support provided by the authors, utilization of the 45 INDELs markers for human identification and complex kinship analysis appears supported. Despite having several reported multiplex systems for X-INDELs loci, review of literature does not reveal any commercial X-INDELs kit available for forensic applications [18] prior to 2021. This has motivated Chen et al. [73] to develop and validate a novel five dye multiplex system with 38 X-INDELs Loci and an Amelogenin locus, named AGCU X-InDel 38 kit for forensic applications, particularly for challenging complex kinship cases and degraded DNA samples. The newly developed kit demonstrated high forensic statistical parameters support when tested on the Han population as well as capable for genotyping severely degraded DNA samples from casework. Nevertheless, the developmental validation of the kit focused only on the East Asian population, and hence, the true potential of the kit on the different populations requires clarification and further assessment.

Progressively, many forensic laboratories are exploring X-STRs analysis on the next-generation sequencing (NGS) or also known as the massive parallel sequencing (MPS), one of the latest technology platforms in DNA profiling [29]. NGS technology has the ability not only to analyze minute quantities of samples (particularly useful for compromised forensic samples) but also to support simultaneous analysis of the different types of markers (e.g. STRs and SNPs) [74]. The MiSeq FGx™ Forensic Genomics System is an example of NGS with Illumina sequencing technology that consists of ForenSeq™DNA Signature Prep kit, MiSeq FGx™ Reagent Kit, MiSeq FGx™ instrument and ForenSeq™ Universal Analysis Software that would enable simultaneous PCR amplification (up to 231 loci) in a single reaction for forensic DNA casework and databasing laboratories [75]. The assay (ForenSeq™DNA Signature Prep kit) contains two DNA primer mixes (A and B) that are targeting Amelogenin, 27 autosomal STRs, 24 Y-STRs, 7 X-STRs, 94 identity informative SNPs, 22 phenotypic SNPs and 56 biogeographical ancestry SNPs. The incorporated 7 X-STRs are similar to those present in the Qiagen Investigator® Argus X-12 QS kit, suggesting potential core loci for X chromosome markers. In another study, Zhang et al. [71] while utilizing the NGS for obtaining information from X-chromosomal analysis (15 X-STRs, 28 X-SNPs and 17 X-INDELs) on the Ion Torrent Personal Genome Machine (PGM) platform reported that the allele percentages among 100 Han individuals ranged between 75.21% (DXS10103) to 92.54% (DXS6803) for the 15 X-STRs analyzed. They further indicated the observation of full concordance between NGS and CE (except at one locus i.e. DXS10103), revealing detail sequence and mutation repeat motifs information that may be useful for forensic cases.

In gist, utilization of NGS for analyzing X-STRs markers can be beneficial in forensic DNA profiling since it provides (a) a higher resolution of the data than that of CE, resulting in (b) higher

PD even for (c) degraded DNA samples. However, the approach may suffer from (a) the higher cost of analysis, (b) the lack of standardization procedure, (c) complexity in the interpretation due to huge amount of bioinformatics data and the fact that the results (d) may reveal sensitive personal data (e.g. genetic anomalies) [76]. Moreover, because the NGS analysis can also be (e) labour intensive and time consuming, its application may not be feasible for routine forensic cases [77]. Taking into account all these facts, the CE-based analysis remains as the current standard tool of choice among forensic practitioners although the rapid development of NGS technology for catering the needs the field of forensic DNA profiling cannot be neglected.

The X-STRs application and discussion on selected cases

Owing to the favorable properties and mode of inheritance, a great deal of studies reported on X-STRs as well as other genetic markers for providing better resolution to the DNA profiling analysis involving biological relationships determination as well as individual identification and trace samples analysis [25]. Considering the importance of these three prong areas, this review will provide suitable discussions on such areas, detailed below.

Biological relationships determination

Butler [9] defined kinship analysis as "DNA evaluations using biological relatives to predict expected genotypes in missing individuals; serves as an indirect form of human identification when no direct reference samples are available". Not only that kinship analysis utilizes information from the biological relatives, it also requires evaluation of genetic pedigrees reflecting the family relationships, prior to the analysis [78]. In this context, LR refers to "how much more likely it is that the DNA evidence would be observed under a hypothesis that the evidence came from people with a specific relationship as opposed to seeing the DNA evidence given a hypothesis that the observed data came from two presumably unrelated people" [78]. Tasks for establishing relatedness among individuals are commonly encountered by forensic scientists, particularly in immigration dispute, as well as missing persons and mass disaster investigations [14]. Because in such cases, primary DNA reference sample may not be available for direct comparison, representation of the results made in the form of statistical evaluation of LR can be more challenging than that of when primary DNA reference sample is available [9]. Hence, it is opined that utilization of sufficient genetic markers (X-STRs) as imperative for providing a better resolution, in terms of DNA evidence support. Table 1 represents the probability of inheriting genetic information (without mutation) for STRs markers on autosomal and sex chromosomes based on specific family relationships (*viz.* mother-son, mother-daughter, father-son, father-daughter, paternal grandfather-grandson, paternal grandmother-granddaughter and maternal grandmother-granddaughter).

Routine paternity trios (when mother, alleged father and the child are present) cases can easily be resolved by autosomal STRs [20]. However, there are situations reported whereby mismatches in autosomal STRs that resulted in an inconclusive conclusion based on the combined paternity index (CPI) [79]. Using three different autosomal STRs

Table 1. The probability of inheriting genetic information (without mutation) for STRs markers on autosomal and sex chromosomes based on specific family relationships, simplified from Butler [81].

Relationship	Autosomal STRs	X-STRs	Y-STRs
Mother-Son	0.5	1.0	n/a
Mother-Daughter	0.5	0.5	n/a
Father-Son	0.5	0.0	1.0
Father-Daughter	0.5	1.0	n/a
Paternal Grandfather-Grandson	0.25	0.0	1.0
Paternal Grandmother-Granddaughter	0.25	1.0	n/a
Maternal Grandmother-Granddaughter	0.25	0.25	n/a

Note: Not applicable (n/a) since females do not have Y-chromosome to transmit to progenies. Values of 0.0 indicate no contribution of genetic information on X-chromosome transmission to progenies from the father/grandfather.

kits (Identifiler, PowerPlex 16 and Genephile G-PlexHuman Autosomal STR) in a paternity trio case, Akhteruzzaman et al. [80] reported one or two mismatches that resulted in inconclusive CPI support for the relationships. The authors further performed the X-STRs analysis and their results conclusively excluded the alleged man as the biological father since 8 out of 13 loci tested were inconsistent with the child. This case has demonstrated the usefulness of X-STRs analysis in paternity investigation, considering that the three different autosomal STRs kit utilized earlier failed to resolve the dispute. Should the alleged man be the biological father, the exact paternal X chromosome (100%) must be established to prove that the daughter is indeed his. This type of association can be seen in Table 1.

In addition, utilization of X-STRs can also be useful in resolving any parent-offspring relationships dispute, involving at least one female (e.g. father-daughter, mother-son and mother-daughter) [82]. In a father– daughter duos relationship, there can only be one allele that can be transmitted from the father to the daughter [25]. Barbaro et al. [41] reported the utilization of X-STRs analysis in a case of deficiency paternity testing, where the mother was available for testing against the hair specimen purportedly from a girl missing for several years. Using the reverse paternity test approach, the authors constructed the allele of the putative father by comparing the X alleles of the mother and sister of the missing girl. Since the DNA profile from the available sister was found "compatible with the reconstructed paternal DNA profile" [41], it can be construed that X-STRs are useful in the investigation of kinship dispute between two sisters or half-sisters based on the paternal X chromosome. However, X-STRs analysis may not be useful for determining a father-son relationship [13] because the father does not transmit his X chromosome to a male offspring [25].With regards to mutational event in duos testing, Chen et al. [83] described two relevant cases (involving a father- daughter and a mother-son investigations, respectively) that utilized both the X-STRs and autosomal STRs analyses. While one autosomal STRs locus mismatch was observed (presumably due to mutation), the X-STRs analysis for both cases resulted in complete matches between the father-daughter and mother-son relationships. The similar situation was also reported by Yu et al. [84] in two mother-son cases. In addition to one or two mismatches in the autosomal STRs analysis due to mutational event, the authors also reported 8 (case 1) and 4 (case 2) X-STRs inconsistencies, hence, disproving the mother-son relationships in both cases. All these four cases described above accentuate the relevance of incorporating additional genetic markers particularly X-STRs in complex kinship investigations since genomic mutations are commonly encountered in autosomal STRs.

Another important aspect in biological relationships determination is resolving maternity issues that may relate to accusations of incest, products of conception from sexual assault as well as abandonment cases [78]. Although mitochondrial DNA analysis could provide maternal inheritance information and can be utilized for forensic human identification maternally [85], this technique is laborious and does not always provide conclusive conclusion [76]. The use of X-STRs analysis in maternity testing appears favorable especially in mother-son kinship investigation [20]. For example, Li et al. [86] reported a maternity case of a woman and a boy that was tested for biological relatedness as part of legal adopting requirements in China. Interestingly, they shared at least one allele at all the 46 autosomal STRs loci analyzed, obtaining a high pairwise-kinship index (parent-child; 6.91×10^8), in support of a mother-son relationship. Upon further testing using X-STRs, 13 out of 20 inconsistencies in X-STRs loci were observed (that included a large step as well as integer to fractional alleles differences), ruling out mutational event and therefore excluding the woman as the biological mother.

Investigation of incestuous cases (e.g. a brother impregnated his sister or a father is both the father and grandfather of a child) is another example where the application of X-STRs analysis can be useful [13, 78]. Gomes et al. [18] alerted the possibility of investigating an incestuous case if a child demonstrated a high number of homozygosities in a paternity testing. It is pertinent to indicate here that, in cases whereby the alleged father of the daughter and the mother (of that daughter) is the same person, both the mother and daughter would share the same X paternal chromosome. In contrary, when the alleged fathers are father and son, both individuals would have different X-chromosomal alleles IBD, resulting in vari-

ation of the X-STRs profile of the child, substantiating higher efficiency of utilizing the X-STRs analysis for paternity testing than that of autosomal STRs [13]. To illustrate this fact, Cosentino et al. [87] reported the high number of shared alleles between the child (a newborn female) and the alleged father (the brother) as well as the underaged mother (the sister) with 99.9999% probability. While the biological father for the siblings (brother and sister) involved in this incestuous case refused to provide DNA sample, and since the siblings are closely related, the authors supported the notion for performing the 12 X-STRs markers that eventually proved that incest accusation [87].

Using the LR for Belarusian and Swedish populations computed by FamLinkX software, Shyla et al. [88] reported on the utilization of X-STRs analysis in resolving complex kinship cases involving putative paternal grandmother. Complete transmission of paternal X chromosome (100%) from a grandmother to a granddaughter (only 25% transmission in autosomal STRs) had resulted in relatively high LR values for determination of familial relatedness, when compared to insufficient resolution following the use of the autosomal STRs profiling and mtDNA sequencing.

Being the most important information in any forensic investigation, individual identification has been routinely performed by comparing the profiles of autosomal STRs [11]. Recently, the use of X-STRs analysis as a form of complementary tool to the routine autosomal STRs has been suggested [14]. This is attributable to the fact that there are cases whereby the interpretation on autosomal STRs profiles alone failed to reveal individuality, largely to the absence of comparison sample as well as for exhibits with mixtures of DNA. In this context, combination of autosomal STRs and X-STRs analyses would substantially increase the discrimination ability that can be useful for forensic investigations [19]. The use of X-STRs analysis may reveal the interrelatedness between the DNA specimens in-questioned with that of the next of kin (e.g. parents and siblings), specifically in cases of missing individuals and/ or when accessibility to the source of DNA for comparison is legally denied [78]. Since, the analysis of X-STRs may also indicate the sex of the persons contributing to the source of DNA (even in mixtures), this trace detection may provide suitable insight in forensic investigation.

Secondly, studies also revealed that the analysis of X-STRs may throw light at diagnosing genetic anomalies experienced by individuals. Among others, genetic anomalies/ diseases that are linked to X chromosome aberration included hemophilia, Duchene muscular dystrophy, Ullrich-Turner syndrome, Klinefelter syndrome, red-green blindness and glucose-6-phosphate dehydrogenase (G6PD) deficiency [13]. Considering the rarity of the anomalies/ diseases and the fact that the X-STRs analysis can potentially reveal this information, its utilization as a potential lead to the forensic investigation may prove useful at narrowing down the search for the potential victim and perpetrators, optimizing the logistic support in the crime scene management.

Spitzer et al. [21] reported the utilization of X-STRs in detecting Klinefelter syndrome from a semen stain of a sexual assault case involving a male perpetrator and a male victim. The semen stain tested had no sperm cell observable under the microscope and the DNA profiling of the non-sperm cell fraction indicated a mixed DNA profiles with an imbalance peak height (twice amount of X chromosome) between X and Y chromosomes, suggesting a possible male-female mixture. Subsequently, the imbalance in Amelogenin locus was consistently observed in another single source DNA profile as well as the reference sample (victim), leading the authors to explore the possible genetic anomalies in the X chromosome and further analyzed the samples using the Qiagen Investigator® Argus X-12 QS kit. The X-STRs results revealed that the presence of two X chromosomes (6 heterozygotes and 6 homozygotes) instead of a single haplotype (typical male) which concluded that the victim had the Klinefelter syndrome (XXY) [21]. In another case, Honda et al. [89] reported on the utilization of X-STRs in identifying the victim (from muscle and bones) of a murder case. The identification of two distinct X alleles in two X-STRs loci confirmed that the victim was suffering from Klinefelter's syndrome. Nonetheless, the fact that review of literature reveals only limited specific forensic cases whereby this particular approach was used, its real potential as forensic intelligence merits further clarification. In doing so, one should observe the ethical issues that can be associated with the utilization of such data for academic purpose.

Population genetics relating to X-STR for human identification

Considering that the DNA profiling in forensic investigation is performed on rather a small fraction of polymorphic regions of the entire human genome, the strength of the DNA evidence is very much dependent on the rarity of the DNA profile obtained in the population [90]. It has to be acknowledged that the forensic DNA profiling relies on the fundamental population genetics principle, similar to those applied in medical field and gene mapping [9]. In this context, Johnston et al. [91] defined population genetics as 'the study of genetic variation within and among populations and the evolutionary factors that explain this variation' with Hardy-Weinberg equilibrium (HWE) as the foundation. Diegoli [14] emphasized on the importance of generating relevant population database to quantify the value of a match between two DNA profiles. Later researchers further accentuated on the fact that the database must be representative of diverse population as well as subpopulations [90]. Since the establishment of population database requires a strong fundamental in genetics, therefore, it is vital to assess the common or rare alleles and genotypes from the representative groups of individuals as a small subset of the population of interest [78, 92].

A scientific standard for forensic genetics was published by Schneider [93], providing important guidelines derived from the National Research Council (NRC) and DNA Commission of the International Society for Forensic Genetics (ISFG) for forensic practitioner in establishing their research work. It is emphasized that ≥500 meioses need to be analyzed for establishing the relevant population genetic parameters (e.g. allele frequencies and mutation rates) for a given population [93]. This proposition is later supported by other researchers in which the minimum of 200 individuals and/or 500 meioses are required for population genetics studies [92]. According to the steps of generating population database suggested by Butler [78], the number and selection of samples must be decided by the laboratory prior to estimating the allele frequencies.

In view of publishing DNA population data, pertinent forensic journals like the International Journal of Legal Medicine and Forensic Science International: Genetics have prescribed specific requirements. With regards to the International Journal of Legal Medicine, the size of population investigated should involve 'at least 200 individuals whereas, regional datasets should contain a minimum of 100 samples' [94]. Additionally, the population data with at least three different markers (e.g. autosomal STRs, Y-STRs and X-STRs) investigated can be accepted for publication in the forms of short communication or original article [95]. As for the Forensic Science International: Genetics, varying requirements are made for X-STRs (12 markers, 500 males), X-SNPs (20 SNPs, 500 males) and X-INDELs (20 INDELs, 500 males) population data [96]. Moreover, the journal further specifies the minimum of 50 unrelated individuals for publication of population data generated by NGS (including X-chromosomal markers). Utilization of forensic statistical parameters is paramount in determining the usefulness of the selected set of genetic markers as well as validating the population data [92]. The parameters are allele frequencies, haplotype frequencies, gene diversity, PD, PIC, heterozygosity, HWE, linkage disequilibrium (LD), power of exclusion (PE), Analysis of Molecular Variance (AMOVA) and F-statistics; Inbreeding Coefficient (F_{IS}), Population Fixation Index (F_{ST}), and Overall Fixation Index (F_{IT}). To perform such analysis, several molecular genetic software programs (e.g. Arlequin and PowerMaker) have been invented to calculate large sample sets with various standard population genetics parameters [97, 98]. However, the X-STRs data would require the evaluation of additional parameters, depending on sex and/or kinship situation [13]. The additional parameters required included mean exclusion chance (MEC) and PD performed for both sexes.

Recently, Lang et al. [99] developed an interactive graphical software for population statistics pertaining to X-STRs called the StatsX (Statistics for X-STR) v2.0 which would enable the calculation of X-STRs forensic statistical parameters which included linkage group with haplotype frequencies in a modest form. In comparison with the ChrX-STR database [100] or other genetic molecular software, the StatsX v2.0 offers input data from Microsoft Excel workbook, evading the step for preparing input files in Arlequin [97] or manually typing each allele or haplotype. However, StatsX v2.0 has limitation in testing the significance of HWE and linkage disequilibrium [99]. Hence, improvement to the existing tools for calculating forensic statistical parameters for X-STRs data would require concerted efforts not

only from forensic DNA expert but also data scientists.

Worldwide populations

Gomes et al. [18] while reviewing the X chromosome use in forensic genetics indicated the relative stagnation in X chromosome forensic research worldwide since 2012, in contrast to its booming period between 2004 to 2011 with the first set of data published in 1999, attributable to several reasons. Firstly, the low number of cases requesting for the X-STRs markers due to the complexity of the analysis when compared with that of Y chromosome, resulting in a relatively high financial cost and the requirements for highly trained analysts [18]. Secondly, due to the complexity of the genetic model of inheritance/ transmission [101], statistical analysis can be difficult to perform and interpret, apart from the fact that such a population study would require a large sample size [102]. Thirdly, the complex evolutionary of genetic model of inheritance/ transmission has also resulted in technical difficulties in establishing specific markers as well as designing suitable primers for amplification [18]. Lastly, the negative perceptions of the scientific communities since X-STRs analysis may reveal sensitive personal information such as physical traits and clinical conditions. Having considered the state of rapidly evolving understanding of molecular biology and the forensically important information/leads that can be derived from X-STRs analysis, application of such a technology supported by solid empirical population data proves necessary, and hence, concerted efforts to overcome all these four limitations must be made.

Owing to its massive population (about 1.41 billion in 2021) [103], China has remarkably reported many X-STRs population data covering mainly on Han ethnic group (e.g.[104, 105]) and several other ethnic minorities such as Sichuan Tibetan (e.g. [106]) and Manchu (e.g.[107]). As a result, active development of various X-STRs multiplexes (e.g. AGCU X19 STR and Goldeneye17X), for forensic and genetic applications, has been reported. Although it is acknowledged that the majority of X-STRs population data are generated from China (e.g. [104, 108]) distribution of the data covering other populations within Asia is under-represented, and to certain extent, unavailable in many countries within this region. As for the other countries within Asia, the available X-STRs data remain limited for certain ethnic groups in Japan (e.g. [49], Korea (e.g. [109]), Malaysia (e.g. [110, 111]), Taiwan (e.g. [112]), Thailand (e.g. [113, 114]) Philippines (e.g. [115, 116]), United Arab Emirates [117], Bangladesh [118], India (e.g. [119]), Iran [120], Iraq (e.g. [121, 122]), East Timor [123] and Sri Lanka (e.g. [124]) and Pakistan (e.g [125]).

While considerable amounts of data have been reported from Germany (e.g. [55]), Italy (e.g. [126]), Spain (e.g. [127]) and Portugal (e.g. [128]), the same for other European countries remains sparse. The countries with sparse X-STRs population data included Albania [122], Austria [129], Belarus (e.g. [130]), Bosnia and Herzegovina [131], Croatia (e.g. [132]), Czech Republic (e.g. [133]), Denmark [134], France [135], Finland (e.g. [136]), Greece [137], Hungary [138], Ireland [135], Latvia [139], Lithuania [122], Poland (e.g. [140]), Serbia [141], Russia (Siberia) [142], Slovak [143], Slovenia [122], Sweden (e.g. [144]) and Turkey (e.g. [122]). Unlike the pattern observed in Asia, population studies related to X-STRs data in the European continent can be perceived as widespread although the frequencies are largely low in majority of the countries. Review of literature further reveals the progression of multiplex systems utilized for such studies, started with the use of Mentype Argus X-UL kit (before 2008), followed by Mentype Argus X-8 kit (2008-2013), GHEP-ISFG Decaplex (2009-2015) and Qiagen Investigator Argus X-12 kit (2012 onwards). As for AGCU X19 STR and Goldeneye17X, utilization of these kits in European countries has never been reported so far.

As for the North and South American continents, the X-STRs population data are very much concentrated towards the United States of America (U.S.) (e.g. [145, 146]), Argentina (e.g. [147]) and Brazil (e.g. [148]). Although the U.S. is a multi-racial population country, the X-STRs population data available in the literature are only reporting on the four major populations *viz.* U.S. Africans, U.S. Asians, U.S. Caucasians and U.S. Hispanics [64, 145]. The fact that the U.S. is a multiracial country and each of the four populations can by itself consists of varying sub-populations, the possible presence of admixture populations can be construed although the specific mention on this aspect is not indicated by the authors. A part from the U.S., Argentina and Brazil, the X-STRs popu-

Table 2. The available X-STRs population data for SEA countries.

Country	Population groups	Population sample size			References
		Total	Male	Female	
Malaysia	Malay (Kuala Lumpur)	283	160	123	[110]
	Indigenous, Peninsular Malaysia; Senoi, Proto-Malay and Negrito	164	68	96	[169]
	Kedayan, Borneo	199	127	72	[111]
Thailand	General	157	116	41	[116]
	Central	391	282	109	[113]
	General	138	138	-	[50]
	Northern	200	200	-	[163]
	Northern	211	61	150	[114]
Philippines	General	115	57	58	[116]
	General (Capital region)	143	143	-	[115]
East Timor	General	149	101	48	[123]

lation data reported within the North America (including Central America and Caribbean) are limited to Mexico (e.g. [149]) and Costa Rica [150]. As for the South America, similar studies are limited to Colombia (e.g. [50]), Ecuador (e.g. [151]) and Peru (e.g. [152]).

As for the African nations that are regarded as having the 'world's deepest population divergences' [153], limited attention is given in the literature on the establishment of X-STRs population data. The existing population data within the African countries included Angola [154], Algeria (e.g. [59]), Cabo Verde [155], Ghana (e.g. [156]), Guinea-Bissau [157], Morocco (e.g. [135]), Mozambique [154], Nigeria [158], Somalia (e.g. [134]), Tunisia [159], Uganda (e.g [154]), Ethiopia [22] and Eritrea [160]. It is noticeable that the African populations being genotyped by researchers (e.g. [50, 135, 161]) for distinguishing genetic differences among the other populations that they studied or in the developmental studies of a new multiplexes, considering that African is a distinct population [161].

Interestingly only one X-STRs study representing the Australia continent [162] is identified in the literature despite its large immigrant populations (Europeans and Asian). The study reported on 298 self-declared Australian Aboriginal males within South Australia and the capital city of Adelaide. According to the authors, the Australian forensic DNA laboratories utilized the existing X-STRs population data (e.g. European and Asian) published by other continents for forensic casework, assuming that no occurrence of post migration genetic drift in their population based on the previously reported Y-STRs study. Therefore, it can be construed that limited population data are available in the Australia continent, particularly on the admixture populations.

It is worth mentioning here that among the many forensic statistical parameters used for describing the worthiness of X-STRs population data, PD, MEC, PIC and haplotype frequencies are considered as the more important parameters. Review of literature reveals variations in the values of these four important parameters among the reported population data. While the PD, MEC, PIC and haplotype frequencies are strongly supportive towards the usefulness of certain population data (e.g. [106, 118]), the same are moderately supportive in the others (e.g. [45, 163]). This can be largely attributed to small sample sizes and the possible presence of unaccounted admixture (e.g. [145, 146]) within the populations studied. The different degrees of statistical supports covering the different populations maybe due to variations in genetic make-up, population stratification [18] and migration flow [164]. Considering that the X-STRs data available in literature are incomplete for many populations worldwide, specific attempts to provide such population data, including for the diverse populations in SEA and admixture populations, may prove necessary. This is because the onus of proof for criminal trials in court is 'beyond any reasonable doubt' [165], and the attempts to provide forensic statistical supports for utilizing X-STRs data in different populations, especially in complex kinship cases, deserve specific forensic consideration.

Southeast Asia

Table 2 depicts the available X-STRs population data for SEA countries. Substantial genetic variations among the many populations in the SEA region have been largely reported in the literature [166] and many authors have attributed such variations to the high diversity in ethnicities, culture and linguistics that are prevailing in the region [167]. For example, the heterogenous population of Malaysia consists of the Malays and Bumiputera (69.8%), Chinese (22.4%), Indians (6.8%) and others minorities/ indigenous populations (1%) [168]. Being a classic example, X-STRs population data for Malaysia are limited to the Malays in Kuala Lumpur alone [110], the sub-ethnicities of indigenous people (Senoi, Proto-Malay and Negrito) in Peninsular Malaysia [169] and one minor ethnicity in Borneo (Kedayan) [111]. Not only the available population data do not represent the diverse population of Malaysia, the one particular study for the indigenous people only utilized 164 participants for representing the three sub-ethnic populations which may not be adequately representative. Interestingly, the only population data reported from East Timor (n=149) population [123] revealed substantial similarity in the forensic statistical support with that of the existing population data in Malaysia. The limitation of small sample size for representing the specific population data for X-STRs was further accentuated in the study report by Salvador et al. [115] for the Philippines population. Despite analyzing a small pool of participants (n=143) to represent the population of more than 100 million people (with over 100 ethnolinguistics groups), the author reported that the X-STRs loci investigated are highly polymorphic with the discovery of six novel sequences. As such, these findings would highly corroborate the substantial diversity of the population, necessitating further studies involving larger sample size.

Regional classification approach was observed in X-STRs population data reported from Thailand, whereby the population studies were performed based on the geographical regions *viz.* Central and Northern Thailand, in consideration of substantial dissimilarity of the Thai populations revealed by previous studies on STRs and SNPs [114]. Interestingly, statistical parameters for DXS8377 in the Northern Thai male population indicated a moderate polymorphism despite the locus being reported as one of the highly polymorphic markers for forensic application [163]. As for the Central Thai population, a high PD was observed among the 12 X-STRs loci analyzed, emphasizing on its usefulness for generating general database [113]. Taking into account that the different regions of Thailand are bordered by other SEA countries (Myanmar, Laos, Malaysia and Cambodia), it is possible that the populations in these regions can be affected by people migrations. This would have an effect on genetic variations as well as the possible presence of admixture within the adjacent geographical locations [113]. Such an assumption was found supported by the closer genetic distance between the Northern Thailand and Taiwanese population, as opposed to the Central Thailand population itself [114]. Curiously, the Thailand X-STRs population data have been selectively used for representing the Asian populations whenever a new multiplex system is developed, and the results are compared with other populations (e.g. Spain, Malawi, Colombia, Germany, Japan and China) [50, 116]. The fact that Thailand is only one of the many countries within Asia, the representativeness of the data from Thailand alone for representing the diverse populations within the continent may prove unjustifiable.

Admixture populations

Admixture population has been described as the "population-level process, whereby gene flow occurs between previously diverged source populations, producing new populations with ancestry from multiple source populations" [170]. It is paramount to indicate here that while a great deal of studies on X-STRs population data have been reported, little emphasis has been given to the population data representing admixture populations. As a matter of fact, limited number of published articles [147, 164, 171–175] reporting on the X-STRs population data of admixture populations are discovered, with none of them representing the Asian populations.

For example, Cortés-Trujillo et al. [172] reported the forensic efficiency of the 12 X-STRs among women in two Mestizo admixture populations and seven Mexican Amerindian (indigenous people of America) groups. Mestizo is a racial classification in the Mexican population that resulted from the admixture between the Native American and European individuals [164], and this racial classification makes up 90% of the

Mexican population [176]. Despite the study [172] reported only on female samples of the Mexican populations, significant pairwise differentiation indicated a close relationship between Amerindian groups and Mestizos. Next, Baeta et al. [164] also conducted X-STRs analysis on the Native American and the admixture group of Mestizo of Central America. The authors described a clear differentiation was observed between these groups with that of the European and African populations, necessitating the establishment of local reference X-STRs database which includes the admixture populations.

Interestingly, while studying the X-STRs data for the heterogenous populations (Europeans, Native Americans and Sub-Saharan Africans) of Argentinian provinces, García et al. [147] indicated that no significant differentiation was observed in the population among the different regions. As such the finding does not appear to support population stratification that can be typically expected when dealing with admixture populations; however, suitable explanation for this phenomenon was not offered by the authors. Hence, further studies for elucidating such a phenomenon deserve consideration.

It has to be acknowledged that interracial marriages are commonly observed throughout the world, making admixture populations as integral parts of the international communities [177]. Since forensic statistical parameters support is an important component in the admissibility of forensic identification using X-STRs analysis and because such statistical support remains generally lacking in many parts of the world, specific attempt to fit into this gap of knowledge shall be encouraged. To do this, a specific focus on the dynamic of multiracial countries (like Malaysia), whereby categorical differentiation in ethnic groups is commonplace, merits scientific and forensic attentions.

Challenges and future insights

Issues on quality assurance and standardization

Having the appropriate quality assurance standard is an important aspect in forensic science, particularly in DNA profiling (for ensuring reliable and accurate data as well as minimizing errors) [178], for providing high quality DNA analysis for the admissibility of evidence in the court of law [179]. Although there are international bodies that work on developing forensic science standards and guidelines, its development in any country relies upon the standards development organization, recognized by the government, for stipulating the relevant quality requirements for supporting the delivery of products or services [180]. Butler and Willis [181] have listed 34 recent prominent published guidance documents (from 2016 to 2019) as references for forensic DNA laboratories. They included eight documents on general quality assurance, seven documents on general procedures and recommendations, four documents on autosomal STRs (Interpretation guidelines, reporting probabilistic genotyping and quality control (QC) for databasing) and four documents on DNA mixture (interpretation, software and internal validations). The remaining documents were three documents on validation (analysis methods, casework material and software for biostatistical calculations), three documents on general reporting (serological examinations, propositions and LR), two documents on mtDNA (interpretations, testimony and reporting), one document on NGS minimal nomenclature requirements, one document on Y-STRs testimony and report, one document on the use of X-STRs in kinship analysis and one document on code of practice and conduct for forensic science providers and practitioner.

The fact that there is only one guidance document available for X-STRs analysis (biostatistical evaluation alone), even for these leading countries (the U.S., the United Kingdom and several Europeans), it is imperative to accentuate the importance of developing the understanding and guidelines on X-STRs analysis for forensic practice as well as the enrichment of the literature. Despite the 34 guidance documents being specifically developed by the leading countries in the DNA research, the fact that science has no physical boundaries, Butler and Willis [181] advocated that such guidelines may also be useful for other regions. Nonetheless, variations may prevail in the accessibility to technologies, facilities, resources and human capital, as well as the quantum of workloads [182] among the different countries. Hence, suitable modifications to the guidance documents may prove necessary for the developing (e.g. Malaysia and Thailand) and the least developed countries (e.g. Cambodia and Myanmar) to embrace the X-STRs technology for providing reliable forensic DNA services in the criminal justice system. As a matter of fact, none of the guid-

ance document available in literature is specifically developed by factoring limitations experience by such countries.

As for the standardization of X-STRs analysis for human identification (including complex kinship cases), two major issues have been identified from the literature *viz.* (1) unavailability of QC checks for databasing and centralized database [18] as well as (2) the appropriate selection of core loci [14]. Besides the importance of QC checks for X-STRs population data, review of literature does not reveal such an aspect relating to uncertainties in the dataset, probably attributable to the lack of centralized database for compilation and storage of X-STRs alleles or haplotype frequencies [18]. Although Szibor et al. [100] attempted to establish an international population database for X-STRs, the database has been relatively stagnant (consisting of only 4 population data) [18] despite a large number of X-STRs population data published in the literature. This situation is largely different from the relatively complete population data for autosomal STRs (STRs for Identity ENFSI Reference Database (STRidER)), Y-STRs (Y Chromosome Haplotype Reference Database (YHRD)) and mtDNA (EDNAP mtDNA Population Database (EMPOP)) [18]. The fact that the data in the reference population are crucial, affecting the statistical evaluation, concerted efforts must be made for the establishment of centralized QC program for X-STRs population data.

While various multiplexes for X-STRs analysis have been reported in the literature, the specific attempt for suggesting the appropriate selection of X-STRs core loci remains lacking, causing substantial confusion among forensic practitioners in selecting the more informative ones that would increase the PD and reduce the adventitious match [183] in forensic caseworks. Importantly, having a standardized selection of X-STRs core loci would enable international compatibility for the exchange of population data, such as that maintained by the expanded Combined DNA Index System (CODIS), as well as comparison and traceability studies. Since participation in international standardization is crucial for ensuring consistency and confidence among the legal communities [180], implementation of analysis of core loci for X-STRs proves forensically relevant.

Knowledge, perception and readiness of forensic and legal practitioners on X-STRs analysis

One of the biggest issues for implementing X-STRs analysis is the fact that the science and technology have to be adequately understood by forensic investigators (scene of crime officers) for analysis, as well as legal practitioners when the evidence is presented in the court of law [184]. To gauge this aspect, specific studies (quantitative and qualitative) that evaluate the knowledge, perception and readiness of these two important groups of people to the applications of X-STRs analysis in human identification must be undertaken. In this context, Panthuen et al. [185] while evaluating 6 DNA laboratories in Thailand concluded that the laboratories were underutilized although they were well equipped with suitable instruments. The underutilization of the DNA services may be due to the fact that at that time 'neither of the 6 forensic DNA laboratories in Bangkok is accredited for forensic DNA analysis by international accreditation bodies nor ISO 17025:2005' although the personnel had positive attitude towards quality assurance standards and accreditations. Another important aspect that may influence the usability of DNA evidence is the ability of forensic scientists to communicate the scientific findings through expert reports and testimonies in the court of law, considering the differences in education, backgrounds, languages and expectations of legal personnel and police investigators [186]. In this regard, Mousseau et al. [187] reported the narrow view on forensic science and its potential among senior police officers in Quebec, Canada, and suggested that closer connections must be bridged among forensic science, policing and security. As such, the authors emphasized on the need of more extensive education for police officers on forensic science. This sentiment is also shared by Crispino et al. [188], calling for paradigm shift in forensic science practice and education for establishing forensic intelligence. As for the lawyers, a qualitative study performed on experience, usage and understanding of DNA evidence in two Australian jurisdictions revealed that they did not fully understand the DNA evidence, especially the statistical evaluations, which may cause major difficulties in assessing the evidence during criminal proceedings. One limiting factor to the accessibility of scientific education is the fact that lawyers are non-science individuals,

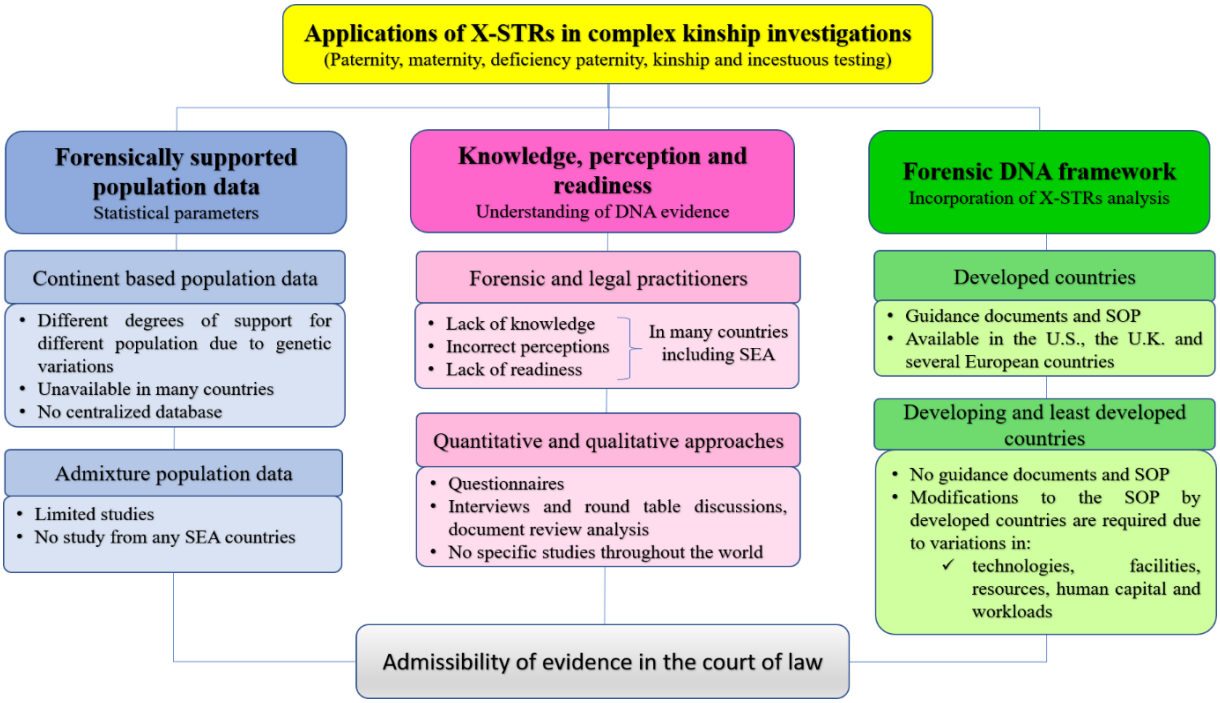

Figure 2. Challenges and future insights into the applications of X-STRs in forensic context

whereas having a bachelor of science degree is the prerequisite for enrolling in forensic science courses especially at master level. Therefore, higher education providers should provide innovative bridging programs (such as post-graduate diploma in forensic science), whereby having a bachelor of science degree is not compulsory for these non-science individuals to enrol (e.g. [189]). Interestingly, while review of literature reveals several studies focusing on the knowledge, perception and readiness of forensic scientists, police investigators and lawyers on DNA profiling *per se* [184, 185, 187, 188], specific studies on the same aspect for X-STRs remains unreported. In addition to the aspects/ factors for the general DNA profiling discussed above, it is anticipated that the utilization of X-STRs analysis may have its own issues. Hence, suitable studies focusing on the knowledge, perception and readiness of forensic and legal practitioners on X-STRs analysis for criminal and civil cases prove necessary.

To ensure the robustness of the DNA profiling analysis performed by a forensic laboratory, the Scientific Working Group on DNA Analysis Methods (SWAGDM) has prescribed specific recommendations for autosomal STR typing [190]. However, specific recommendations for X-STRs analysis by SWAGDM remains unavailable. In tandem with the increasing number of cases involving X-STRs analyses worldwide, specific recommendations have been made by the DNA Commission of the ISFG that include *inter alia* (1) its use as supplementary analysis in paternity and kinship cases, (2) the use of haplotype frequencies in likelihood calculations and (3) involvement of linkage equilibrium tests for generating population frequency data for X-chromosomal marker multiplex [19]. Moreover, the Paternity Testing Commission (PTC) of the ISFG has suggested the use of specific biostatistical parameters for investigating paternity cases revolving around several recommendations [191]. The recommendations included (1) clarifying and defining basic concepts of genetic hypotheses and calculation to produce valid paternity index and (2) addressing issues related to population genetics and special circumstances (deficiency/reconstruction and immigration cases). In addition, the same authors further accentuated (3) the importance of having strategies for the admissibility of genetic evidence for paternity cases as well as (4) the necessary documentation, reporting details and assumptions underlying calculations made for paternity analysis. By integrating recommendations made by these scientific authorities (ISFG, SWAGDM and PTC) a number of countries have developed their very own SOP/ guideline for dealing with cases that may require the use of autosomal, Y-STRs and X-

STRs analyses [39, 79]. However, review of literature reveals that such SOP may require improvements. For example, while the SOP developed by the Central Forensic Science Laboratory, Directorate of Forensic Science Services, India depicts clearly the process for performing the analysis of autosomal STRs, Y-STRs and X-STRs, guidelines for interpretation of such analysis are only provided for the autosomal STRs and Y-STRs alone [192]. Such a situation may limit the use of X-STRs analysis for forensic practical casework. Interestingly, besides having the relatively comprehensive forensic services, similar SOP/ guideline for incorporating the use of autosomal, Y-STRs and X-STRs analyses for forensic caseworks remains unavailable in many Asian countries including Malaysia. Nonetheless, the mere adoption of the SOP/ guideline crafted by developed countries may not be appropriate for Malaysia due to differences in workloads, budgetary constraints, facilities and expertise as well as the readiness of the legal systems.

It has to be mentioned here that, despite being a qualitative approach, the round table discussion (RTD) appears useful for gaining insight and understanding from the relevant stakeholders, in view of developing suitable SOP/ guideline [193]. The idea of having RTD for developing SOP is not only applicable to social sciences alone but also for hardcore scientific areas like forensic genetics for mass disasters victim identification (DVI). In this context, it is pertinent to quote that 'The idea to work on DNA-specific recommendations was born after a round table discussion dealing with the 2004 tsunami disaster in south east Asia' [194]. The authors indicated that their recommendations included training forensic geneticists for DVI and active response planning, as well as covering the DNA testing for criminal casework and kinship investigations [194]. Taking into account the advantages of RTD and the fact that suitable SOP/guideline for incorporating X-STRs analysis within the overall framework of the forensic DNA services in Malaysia remains lacking, performing such a qualitative measure involving the various forensic stakeholders (e.g. forensic scientists, crime scene investigators, lawyers and academics) in developing such SOP may prove pertinent. Figure 2 represents the challenges and future insights into the applications of X-STRs in forensic context.

Conclusion

Utilization of X-STRs analysis in complex kinship identification has been strongly advocated in literature. However, concerted efforts for establishing forensic statistical support for the diverse populations in Asia (especially the admixture populations), standardizing core loci and procedure, improving the knowledge among practitioners as well as developing suitable SOP for incorporating X-STRs analysis in the overall DNA profiling framework in developing countries like Malaysia, prove necessary. A specific focus must also be drawn to the establishment of centralized X-STRs database for enabling effective sharing of forensic intelligence, especially in dealing with transborder forensic investigations.

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