

# Improving human forensics through advances in genetics, genomics and molecular biology

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**Abstract** | Forensic DNA profiling currently allows the identification of persons already known to investigating authorities. Recent advances have produced new types of genetic markers with the potential to overcome some important limitations of current DNA profiling methods. Moreover, other developments are enabling completely new kinds of forensically relevant information to be extracted from biological samples. These include new molecular approaches for finding individuals previously unknown to investigators, and new molecular methods to support links between forensic sample donors and criminal acts. Such advances in genetics, genomics and molecular biology are likely to improve human forensic case work in the near future.

## Short tandem repeat

A DNA sequence containing a variable number (typically  $\leq 50$ ) of tandemly repeated short (2–6 bp) sequence motifs, such as (GATA) $_n$ . Forensically used STRs are usually tetranucleotide repeats, which have few stutter artefacts (see below).

## Forensic DNA databases

National databases held by the police or the justice system of defined short tandem repeat profiles, usually from persons convicted of a defined crime.

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DNA profiling with sets of highly polymorphic autosomal short tandem repeat (STR) markers has now been applied in various aspects of human identification in forensic investigations for nearly 20 years, and the concept and details have been summarized in a previous article<sup>1</sup>. In brief, autosomal STR profiles are generated from biological materials found at crime scenes and compared with profiles of known suspects identified by police investigations or included in national forensic DNA databases. With statistical support, a profile match provides strong evidence for individual identification (except for monozygotic twins, 0.35% in most Western countries<sup>2</sup>), whereas a mismatch does not. Highly similar STR profiles may indicate the involvement of closely related individuals and warrant a more complex statistical interpretation<sup>3</sup>. STR profiling is also used for disaster victim identification (DVI), where matching profiles between human remains and antemortem samples belonging to the victim, or profile similarities with genotyped relatives, provide identification evidence<sup>1</sup>. Moreover, STR profiling is commonly applied to determine kinship, including paternity testing<sup>4</sup>. The use of STR profiling to identify perpetrators, disaster victims and family members has proven to be very successful. Equally important, in numerous cases individuals could be excluded from involvement in crimes or family issues on the basis of STR profiles. During the past few years, significant progress has been made in solving some technical challenges associated with forensic STR profiling.

These include the improved ability to analyse degraded DNA and low amounts of DNA, an increase in discrimination power, and the application of STR profiling for familial searching (BOX 1).

Even with these technical advances in DNA profiling using autosomal STRs, important challenges remain for the use of genetic approaches in human forensic investigations. This Review aims to discuss how recent advances, driven by research progress in fields such as human genetics, genomics and molecular biology are tackling these challenges. We begin by discussing the use of new types of genetic marker — specifically single nucleotide polymorphisms (SNPs) — for universal human identification, which have the potential to overcome some important remaining technical problems of STR profiling but also bring new challenges. We then discuss progress in identifying male DNA components in mixed male/female samples, an area that presents serious problems for autosomal STR profiling. The use of Y-chromosomal STRs (Y-STRs), particularly those that rapidly mutate, promises improved identification in such cases. Comparative DNA profiling — using any genetic marker — can only identify persons already known to the investigating authorities. Aiming to overcome this general limitation, we discuss how recent advances in human genetics and genomics are expected to provide investigative leads for finding persons previously unknown to the investigators; for example, by inferring biogeographic ancestry and externally visible

Box 1 | **Developments and improvements in STR-based human identification**

**Single nucleotide polymorphisms**

DNA sequence variation concerning a single site (base pair) in the genome. The polymorphism is usually a substitution, but can sometimes be a single base pair insertion or deletion.

**Biogeographic ancestry**

A concept of lineage that looks at kinship and descent based on biogeography, a combination of biology and geography.

**Low template DNA**

The availability of just a few DNA molecules for DNA profiling.

**Allele drop-in**

Addition of (typically) one or two alleles to a DNA profile, owing to contamination.

**Allele drop-out**

Loss of one or both alleles in a DNA profile, owing to stochastic failure of PCR amplification, usually when the number of template molecules is small.

**Heterozygote peak imbalance**

The proportion of the two alleles of a heterozygote genotype, expressed as the area of the smaller peak divided by the area of the larger peak in an electropherogram.

**PCR amplicon**

DNA that is generated by PCR amplification.

**Stutter artefacts**

Artefacts that occur by DNA-replication slippage during the PCR amplification of STRs. Most stutter artefacts seen with fluorescence-based STR analysis are one repeat shorter than the true allele.

**Multiplex genotyping**

Simultaneous analysis of multiple genetic loci.

**Match probability**

The chance of two unrelated individuals sharing a DNA profile.

**Dealing with sample degradation**

The sizes of the PCR amplicons of the forensically used short tandem repeats (STRs) included in commercial kits can be as large as 500 bp. This can lead to genotyping failures when working with fragmented DNA from degraded samples, which is commonly encountered in crime scene investigation and disaster victim identification (DVI). With the recent introduction of commercial miniSTR kits, fragment sizes have been reduced mostly below 200 bp<sup>113</sup>, and the use of miniSTRs is expected to substantially increase the efficiency of STR-based human identification<sup>114</sup>. However, current miniSTR fragment sizes may still be too large for the successful analysis of heavily fragmented DNA found in some forensic cases, especially in DVI, where very short amplicons of ~50 bp are needed. In general, such short PCR amplicons are not achievable with STRs owing to the length of the repetitive sequence they include.

**Increasing sensitivity**

Progress in forensic DNA technologies goes along with increased demands from investigating bodies to push the technical limits of forensic STR profiling. Therefore, scientific studies have tested non-conventional biological materials for successful STR profiling, such as touched objects<sup>115</sup>, including ammunition<sup>116</sup>, used lip stick<sup>117</sup>, and even food that has been bitten into<sup>118</sup>. However, in many such cases the amount of DNA available for STR profiling is very low, and the analysis of such low template DNA introduces problems such as allele drop-in and drop-out, increased heterozygote peak imbalance, and high levels of stutter artefacts. These factors result in difficulties in individual STR profile determination, particularly from mixed samples. These issues have caused controversies and ongoing discussions on the use of low amounts of DNA in forensic analysis, and the need for guidelines for genetic analysis, data interpretation and practical implementation<sup>119–121</sup>. These discussions have prompted arguments on the value of such DNA evidence in criminal proceedings<sup>122–125</sup>. The recently introduced miniSTRs have increased the success of analysing low amounts of even degraded DNA<sup>113</sup>. However, pushing the sensitivity limits of forensic STR profiling also increases the risk of detecting DNA contamination; recent incidents with manufacturer contamination of swabs used for crime sample collection<sup>126</sup> have resulted in new guidelines for preventing and detecting DNA contamination<sup>127</sup>.

**Improving accuracy**

Until recently, a European standard set (ESS) of seven STR loci was common to all European national forensic DNA databases<sup>128</sup>; this is considerably less than the 13 STRs applied in the United States and in many other countries. It is known that using insufficient numbers of STRs can lead to false-positive matches<sup>129</sup>. Consequently, pan-European efforts to fight crime using STRs were limited, especially when considering partial STR profiles, which became a serious issue with the recent establishment of regulations on sharing forensic STR profiles among European countries<sup>130</sup>. Therefore, the Council of the European Union recently adopted a resolution to add five additional autosomal STRs to the current ESS<sup>131</sup>, which have now been implemented into new commercial kits<sup>132,133</sup>.

**Familial searching**

There are ongoing discussions as to whether forensic STR profiling should be used for finding unknown persons through their relatives who's DNA profiles already are included in forensic DNA databases, using the principle of STR allele sharing between biological relatives. Although forensic cases have already been solved using this indirect approach, referred to as familial searching, there are reservations regarding its practical application both from the scientific point of view and, perhaps of even more concern, from a legal perspective. These issues need to be resolved before this method is implemented more widely (for a recent review, see REF. 134).

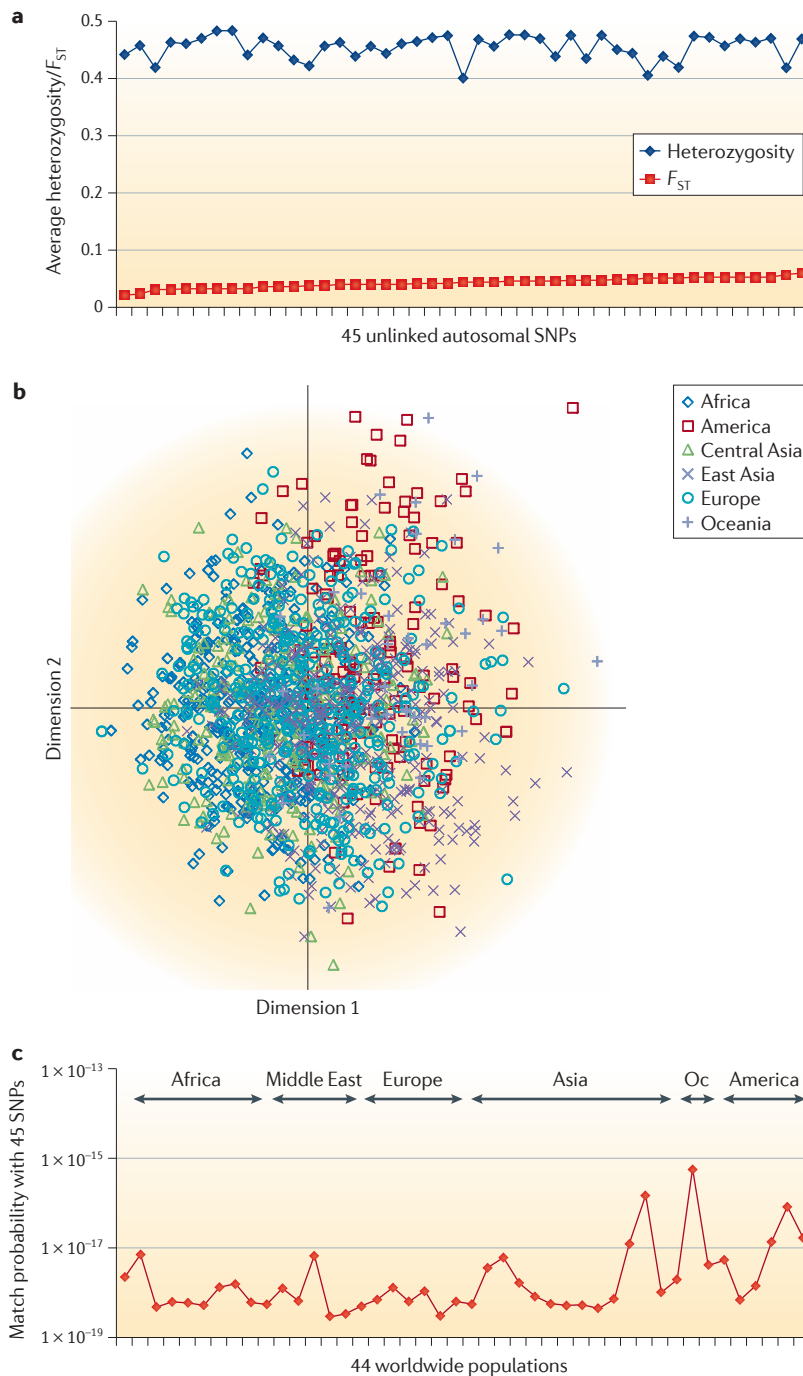
characteristics (EVCs) from DNA. We conclude by addressing how novel genomic and other molecular approaches provide investigators with new intelligence that could fill missing links between DNA-identified sample donors and specific criminal acts. New molecular approaches are being developed for this purpose, such as determining the cellular nature of a crime scene sample and estimating the time of sample deposition at the crime scene.

**New tools for identifying known persons**

*Using autosomal SNPs for human identification.* Many of the technical difficulties with the forensic use of STRs do not exist for SNPs. The use of very short PCR amplicons (for example, 50 nucleotides or less) is essential for the successful analysis of low amounts of highly degraded DNA. This is impossible with highly polymorphic STRs because of their repetitive sequence (BOX 1). However, as SNPs reflect single base changes, very short PCR amplicons can be employed, enabling

successful SNP profiling from degraded DNA in samples from which STR profiles cannot be obtained<sup>5,6</sup>. Furthermore, because SNP variation does not involve repetitive sequences, SNP profiling avoids stutter artefacts that complicate STR profile interpretation, especially when low amounts of DNA are analysed.

The use of autosomal SNPs for human individual identification in forensic investigations (see below for the use of SNPs for inferring biogeographic ancestry) also presents challenges. Obviously, bi-allelic SNPs are less polymorphic than multi-allelic STRs. Therefore SNPs are less informative in the analysis of mixtures of DNA from multiple individuals, although the use of a higher number of SNPs relative to STRs<sup>5,6</sup>, and/or perhaps the use of tri-allelic SNPs<sup>7</sup>, combined with multiplex genotyping technologies<sup>5,6</sup> may compensate for this effect. It has already been demonstrated that 20–50 ascertained autosomal SNPs could reach match probabilities similar to those obtained with 10–15 forensically used STRs<sup>5,6,8</sup>.



**Figure 1 | Autosomal SNPs suitable for universal human identification.** Forty-five unlinked autosomal SNPs were ascertained by screening more than 500 candidate SNPs in 44 worldwide populations, as described elsewhere<sup>8</sup>. **a** | These 45 ascertained SNPs have high levels of heterozygosity and low levels of population differentiation as expressed by  $F_{ST}$  (the proportion of genetic diversity found between populations relative to the amount within populations), and are therefore suitable for universal human identification purposes. **b** | These 45 SNPs do not detect human population substructure, as illustrated by the random positioning of 1,635 individuals from the 44 worldwide populations in a two-dimensional plot. The plot is derived from multidimensional scaling analyses of pairwise identity-by-state distances, which were averaged across all SNPs. See FIG. 2 for the amount of population substructure detectable with genome-wide SNPs and with ancestry-sensitive SNPs. **c** | These 45 SNPs reveal match probabilities — shown in log scale for each of the 44 worldwide populations, considering all SNPs — that are comparable with match probabilities from sets of forensically used short tandem repeats. Oc, Oceania.

Another challenge is to develop SNPs for universal human identification with similar reliability in individuals of diverse biogeographic ancestries. Two population genetic aspects are important in this respect: the preferred SNPs should have similarly high degrees of allelic diversity in worldwide populations, and should have low degrees of population differentiation across worldwide populations<sup>9</sup>. The former characteristic is important to reach similarly high matching probabilities among people of different biogeographic ancestries; the latter is crucial to avoid the effects of population substructure when estimating match probabilities. Publicly available human genomic resources, such as the International HapMap Project, are useful to ascertain SNPs from multiple populations, but do not provide sufficient worldwide data coverage. FIGURE 1 provides an example of 45 unlinked autosomal SNPs suitable for universal human identification that were ascertained according to the two aforementioned population genetic criteria, using genotype data from a large number of candidate SNPs in a large number of globally dispersed human populations<sup>8</sup>. Multiplex genotyping assays for these SNPs have been developed recently<sup>10</sup>, but are not yet commercially available. In principle, large numbers of SNPs, such as those available via commercial high-resolution SNP microarrays that contain hundreds of thousands of markers, are useful for universal individual identification. However, the current SNP microarrays require substantial amounts of genomic DNA, which must not be heavily fragmented — a requirement that is often impossible with crime scene samples.

Besides technical and biological issues, another important aspect makes the potential general use of autosomal SNPs for crime scene investigation unpopular: all existing national forensic DNA databases are STR-based. Furthermore, legislation in many countries prevents retention of DNA samples from convicted offenders. Hence, introducing SNP-based human identification for routine forensic case work would, for many countries, involve starting from scratch in establishing forensic DNA databases, a prospect that is not very appealing. However, countries that do keep DNA samples, and those which have not yet started to establish a forensic DNA database, may consider the use of SNPs for forensic identifications once commercial kits with suitable markers become available.

A notable exception where no pre-existing DNA databases are required is the identification of missing persons, including DVI. Here, DNA profiles from antemortem samples of victims, or reference samples from the victims' relatives, are *de novo* collected and genotyped together with the victims' remains<sup>11</sup>. The replacement of STRs by SNPs is therefore more straightforward and also highly beneficial, as DNA is usually highly degraded in such cases. SNP markers for DVI have been applied in various instances, including after the terrorist attacks of 11 September 2001 on the World Trade Center in New York City<sup>12</sup>. Finally, SNPs, because of their 100,000-times lower mutation rate compared with STRs, are superior for kinship testing<sup>13</sup> and may replace STRs for such a purpose once commercial kits become available.

**Using Y-STRs for male identification.** Another forensic field in which new DNA markers promise to improve human identification is mixed-stain analysis. The ability to specifically identify male individuals in cases of sexual assault, where usually mixed stains are analysed that contain DNA from both the male perpetrator and (often in large excess) the female victim, is extremely important. However, autosomal STR profiling is often not successful in such cases because of preferential PCR amplification of the excess female component<sup>14</sup>. Haplotypes from sets of non-recombining male-specific Y-STRs have been used for male identification since the late 1990s<sup>15</sup>, especially once commercial kits containing up to 17 well-defined Y-STRs<sup>16</sup> became available. Progress has been made in developing frequency databases for these particular Y-STRs needed for statistical interpretation of Y-STR profile matches. Y-STR haplotype databases must be larger than frequency databases for autosomal STRs owing to the non-recombining nature of the male-specific part of the Y chromosome. The largest Y-STR database is the publically available [Y Chromosome Haplotype Reference Database \(YHRD\)](#)<sup>17</sup>, which currently includes Y-STR profiles from nearly 100,000 men from over 700 populations of more than 100 worldwide countries.

The particular Y-STR markers currently used in forensics allow the identification of groups of paternally related men (paternal lineages) in most human populations with a high level of resolution<sup>14</sup>. Exceptions include those populations, such as the Finns<sup>18</sup>, that have recently undergone bottleneck events or founder events with subsequent expansions. Adding additional Y-STRs to the current sets can improve the level of paternal lineage differentiation, as was recently shown on a worldwide scale<sup>19</sup> and also in Finns<sup>20</sup>. Hence, more markers should be integrated into future commercial Y-STR kits; suitable Y-STRs are available, for instance from a comprehensive survey study<sup>21</sup>.

Overall, current Y-STR sets provide suitable tools for excluding males from involvement in crime cases such as sexual assault. However, there is ongoing discussion about how to use frequency databases for estimating the statistical weight of a Y-STR haplotype match<sup>22–24</sup>. In the absence of sufficient empirical data, it is difficult to know how reliable Y-STR haplotype frequencies obtained from existing Y-STR databases are, because they contain only unrelated males. It is expected that the uncertainty about the true Y-STR haplotype frequency is larger in rural areas, where more related men are expected to be found in a specific region, and is smaller in metropolitan areas. Ideally, Y-STR haplotype frequency databases should be established from randomly chosen men, including related and unrelated individuals, to reflect the amount of male population substructure in a region.

A general limitation of the particular Y-STR markers currently applied in forensics is that, unlike autosomal STR profiling, assessment of the weight of evidence is limited for a single contributor as closely related males can not be excluded. This is because of the low mutation rates of the currently used Y-STRs, which are in the

order of a few mutations per marker every 1,000 generations<sup>25</sup>. Such a low mutation rate makes it unlikely that a mutation event occurs between paternally closely related men. However, a recent systematic study that investigated the mutation rate of 186 Y-STRs<sup>26</sup> identified 13 markers with much higher mutation rates of a few mutations per marker every 100 generations. With these 13 rapidly mutating (RM) Y-STRs, it was possible to differentiate >70% of close and distantly related males, whereas the 17 Y-STRs currently used in forensics only differentiated 13% of male relatives from the same samples<sup>26</sup>. The RM Y-STRs proved particularly useful for distinguishing between closely related males (separated by 1–5 male generations), for which the currently used Y-STRs provided almost no differentiation<sup>26</sup>. RM Y-STRs are expected to be useful in all cases in which the currently used Y-STRs did not determine whether a given man or any of his paternal relatives was involved; that is, all cases with matches in current Y-STRs. They also represent an ideal tool for special cases of sexual assault with multiple related male perpetrators involved, where autosomal STR-profiling and current Y-STRs are not usually informative.

We expect that RM Y-STRs will revolutionize Y-chromosomal applications in forensic analysis, from paternal lineage differentiation to male individualization. Although not formally tested yet, RM Y-STRs, because of their higher mutation rate, are likely to provide higher paternal lineage differentiation than currently used Y-STRs that have much lower mutation rates. One could even speculate that, if commercial kits become available, RM Y-STRs may replace current Y-STR sets in crime scene investigation and those DVI cases with antemortem samples because of their value for male individualization. However, in other forensic applications, such as kinship testing or DVI cases that involve testing relatives, Y-STRs with lower mutation rates (such as those currently in use) will still be preferred<sup>27</sup>, because mutations complicate family reconstructions.

### New tools for finding unknown persons

In general, the current comparative approach of forensic DNA profiling — irrespective of which type of genetic marker is used — only allows the identification of persons who are already known to the investigating authorities. In the absence of a universal DNA profile database, without large enough forensic DNA databases, without efficient policing, and with the ethical, legal and economic problems of DNA mass screenings, one promising approach remains for guiding police investigations to find unknown persons. This approach is known as forensic DNA phenotyping (FDP) and includes the inference of information on biogeographic ancestry and EVCs directly from a DNA sample. For crime scenes, FDP is expected to reduce the pool of potential suspects and so concentrate and guide police investigations to find the previously unknown perpetrators<sup>28</sup>. FDP may also be helpful in missing person identification, including DVI, to provide leads for finding relevant antemortem samples or relatives. Clearly, FDP is not free of ethical issues and also needs legislative support, and both issues

#### Population differentiation

Populations that differ to a certain extent in their genetic characteristics.

#### Haplotype

A specific Y chromosome or mitochondrial (mt) type defined by the combination of genotypes of more rapidly evolving markers, usually STRs on the Y chromosome for Y haplotypes, and the mtDNA sequences — including rapidly and slowly evolving sites — for mtDNA haplotypes.

#### Bottleneck event

A marked reduction in population size followed by the survival and expansion of a small, random sample of the original population. It often results in the loss of genetic variation and more frequent matings among closely related individuals.

#### Founder event

A situation in which a new population is founded by a small number of incoming individuals. Similar to a bottleneck, the founder effect severely reduces genetic diversity, increasing the effect of random drift.

**Box 2 | Ethical aspects of forensic DNA phenotyping**

Methods to predict the physical appearance of crime suspects through genetic analysis raised ethical concerns 5 or 6 years ago when stories of their use by law enforcement first surfaced<sup>135</sup>. In Europe, these concerns highlighted potential risks to privacy and autonomy if the revealed traits were previously unknown to, or had been concealed by, the subject<sup>136</sup>. In addition, British and US publications voiced concern that law enforcement might misconstrue the probabilistic, context-dependent nature of biogeographical ancestry information and use it not to focus an investigation but to justify targeting racially identified populations<sup>136–138</sup>.

Scant information is available on police use of these methods, but what is known seems to allay fears of ethical risks. The only national law on the topic addresses privacy concerns by limiting analysis to externally perceptible traits evident since birth and biogeographic ancestry inference<sup>139</sup>. In addition, in one known case, the use of forensic DNA phenotyping seems to have mitigated rather than encouraged ethnic bias<sup>140</sup>. In this instance, biogeographical ancestry DNA testing indicated that the murderer of a 16-year-old Dutch girl was of western European descent, a finding that challenged town residents' suspicions that the perpetrator resided in a nearby hostel for asylum seekers from the Middle East and North Africa and calmed a volatile situation. These cases support claims that government regulation informed by public discussion can limit risks associated with DNA phenotyping methods.

However, ethicists emphasize the limits of regulation<sup>141</sup>. As the history of laws requiring DNA from an ever-expanding list of suspects and criminals demonstrates, restrictive regulations can always be loosened. Furthermore, although some risks — such as those determined by which traits can be analysed — are amenable to regulation, others that are related to interpreting results are not. To forestall misinterpreting biogeographical ancestry as race, researchers emphasize that ancestry results pertain only to geography, not physical appearance<sup>28</sup>. Distinguishing appearance from ancestry goes to the heart of the problem, but still might not deter inaccurate inferences about race. This is troubling, especially in law enforcement, in light of research suggesting that simply identifying someone by race can activate negative attitudes<sup>142</sup>. Ethicists emphasize education, active monitoring and guidelines for interpretation to promote beneficial use of these methods.

**Genetic clusters and clines**

Populations in close geographic proximity that have similar genetic characteristics (clusters), or populations that show a genetic frequency gradient that correlates with the geographic distances separating them (clines).

**Effective population size**

The number of breeding individuals of an idealized population that has the same properties with respect to genetic drift as does the actual population in question.

**Genetic drift**

The stochastic fluctuation of allele frequencies in a population owing to chance variations in the contribution of each individual to the next generation.

**Residence pattern**

Referring to conventional rules or patterns of behaviour concerning the place a couple lives after marriage.

**Haplogroup**

A specific Y chromosome or mitochondrial type defined by the combination of genotypes of slowly evolving binary markers usually SNPs on the Y chromosome or mtDNA, respectively.

**Hypervariable region**

Part of mitochondrial DNA that is non-coding and therefore accumulates variation more than the coding parts.

are addressed in BOX 2 and BOX 3, respectively. It should be stressed here that FDP should only be applied to unknown samples for intelligence work, in a similar way to how eyewitness statements are used today<sup>28</sup>. However, in contrast to eyewitness statements, which seem to have serious error rates<sup>29</sup>, the value of DNA-derived EVC and ancestry information can be statistically supported.

**Inferring biogeographic ancestry from DNA.** From a theoretical perspective, exactly what is revealed by reconstructing a person's biogeographic ancestry from DNA information is unclear<sup>30,31</sup>. What it will certainly not reveal is where all biological ancestors of a given person came from. For instance, going back in time for just ten generations, we all have 1,024 pedigree ancestors, many of whom are related and shared among different individuals<sup>32,33</sup>. This 'Malthusian parameter of ascent', combined with the fact that we are a relatively young species and that our ancestors arrived in the various regions of the world relatively recently, explains the globally very complex and mosaic-like landscape of genetic clusters and clines<sup>34</sup>, which strongly influences any DNA-based inference of biogeographic ancestry. Obviously, clear exceptions exist for a person's strict paternal ancestor, which is always exactly one male per generation and traceable using Y chromosome DNA information, and the strict maternal ancestor, always exactly one female per generation and traceable using mitochondrial (mt) DNA information. It is therefore not surprising that these paternal and maternal lineage markers have been identified as valuable sources of geographic information<sup>35</sup>. Population genetic features such as their reduced effective population size and lack of recombination make them especially prone to genetic drift effects<sup>35,36</sup>, as well as the potential effects of cultural traits such as residence pattern<sup>37,38</sup>.

Large numbers of Y-SNPs are available for which we have a good understanding of their phylogenetic relationships, a commonly agreed Y haplogroup nomenclature<sup>39</sup>, and reasonably good knowledge of their global frequency distributions<sup>35</sup>. These factors make it tempting to use Y-SNPs for inferring paternal biogeographic ancestry. Global Y chromosome diversity can be captured by genotyping 20–30 Y-SNPs that define the 20 major Y chromosome haplogroups which have differential frequency distributions around the world<sup>35,40</sup>. Some of these Y haplogroups are restricted to a single continent: A and B are nearly exclusively found among sub-Saharan Africans; H is almost exclusively found on the Indian sub-continent (and among Roma); and M is almost exclusively found in Oceania. Other Y haplogroups such as R and N are seen across vast areas of Eurasia<sup>35</sup>. Some geographic knowledge can be obtained from Y-STRs using global knowledge about haplotype distribution in geo-referenced databases such as YHRD; however, most Y-STR haplotypes are too rare to carry geographic information.

Alternatively, maternal biogeographic ancestry can be inferred from informative mtDNA data. Although the hypervariable region is commonly sequenced for forensic identification purposes, it does not provide enough information for geographic assignment of some mtDNA haplogroups. Recent progress in whole mtDNA sequencing has provided increased understanding about the mtDNA phylogeny and revealed a large number of different mtDNA haplogroups<sup>41</sup>. Many mtDNA haplogroups show restricted continental distributions, such as haplogroup L to Africa, V to Europe and the Middle East, or P and Q to Oceania<sup>35</sup>. Genotyping approximately 20–30 coding mtDNA SNPs is likely to allow the definition of most major mtDNA haplogroups with strong frequency differences between geographic regions<sup>42</sup>.

**Box 3 | Legal aspects of forensic DNA phenotyping**

The legal framework for determining biogeographical ancestry or visible traits for criminal investigation — forensic DNA phenotyping (FDP)<sup>139</sup> — is underdeveloped. Almost all countries allow classic DNA profiling using short tandem repeat markers, but many countries stipulate that only ‘non-coding’ regions can be used for DNA profiling; that is, regions that are not known to provide for any functional properties of an organism<sup>143</sup>. However, some non-coding markers are associated with visible traits<sup>76</sup> and others can provide information on biogeographic ancestry<sup>144</sup>. Hence, non-coding markers in DNA from suspects’ samples could be used for FDP, although this would violate the spirit of these laws. Furthermore, DNA profiling laws usually only cover samples taken from convicts, suspects or volunteers, and say nothing about analysing crime-scene stains, to which FDP will be applied.

Common-law systems, such as those in the United Kingdom and the United States, may allow new criminal investigation methods unless the law provides otherwise; this suggests that FDP carried out on crime-scene stains is allowed in these jurisdictions unless specifically prohibited by law<sup>139,145</sup>. Civil-law systems, such as those in continental European countries, only allow investigative powers on a specific statutory basis, which *de facto* prohibits FDP unless specifically provided by legislation. The Netherlands is the only country to explicitly allow the use of FDP to determine gender or race (biogeographic ancestry) in legislation; once the technology has sufficiently matured, specific externally perceptible traits can also be allowed, but only traits visible from birth and unrelated to disease<sup>139</sup>. The US state of Texas implicitly allows FDP, even for genetic diseases<sup>145</sup>. Some other jurisdictions, such as Germany and the US states of Indiana, Rhode Island and Wyoming, explicitly prohibit deriving physical traits (other than gender) or propensities for disease from DNA; Vermont and Utah only exclude the determination of genetic diseases<sup>139,145</sup>. The legislation of most other jurisdictions is silent about FDP.

As scientific knowledge and technology are moving forward, legislatures may consider passing specific legislation on FDP, after societal and parliamentary debates on its acceptability. Regulatory issues to take into account are non-discrimination (particularly salient as FDP is most useful for tracing suspects from minority groups<sup>137</sup>), privacy and data protection, the ‘right not to know’, and preventing ‘slippery slopes’. These are serious issues, but their particular relevance to FDP should not be overestimated<sup>139</sup>. FDP can usefully contribute to criminal investigation, to include or exclude groups for further investigation, fitting the trend in criminal law towards intelligence-led policing<sup>146</sup>.

Unfortunately, effective multiplex genotyping tools for the reconstruction of paternal and maternal biogeographic ancestry based on Y and mtDNA SNPs are scarce<sup>43,44</sup>. Furthermore, these markers are susceptible to issues that make the accuracy of DNA-based biogeographic ancestry inference uncertain in some contexts<sup>45</sup> (BOX 4). The most important factor here is sex-biased genetic admixture, where a person’s paternal ancestors come from a different geographic region to the maternal ancestors. Well-known examples in which this applies to entire geographic regions (although for different underlying reasons) come from South America<sup>44</sup> and Oceania<sup>46</sup>. However, because it can never be excluded *a priori* that an unknown sample donor is of mixed biogeographic ancestry, especially in countries where continental groups are now living next to each other, informative sets of Y-chromosomal and mtDNA markers need to be combined together with those from the autosomes for a more accurate DNA-based inference of biogeographic ancestry.

Based on large, genome-wide sets of autosomal SNPs, which recently became available through commercial microarrays, biogeographic ancestry has now been studied in the context of a wide range of geographical distances. Global studies involving all major continental regions<sup>47,48</sup> (FIG. 2a,b) and studies within (sub)continents — that is, Africa<sup>49</sup>, India<sup>50</sup>, Asia<sup>51</sup> and Europe<sup>52</sup> (FIG. 2c) — revealed genome-wide genetic population substructure on different levels as assessed using various statistical approaches (BOX 4). Of course, this does not suggest that such geographic resolution can be achieved in all possible cases. Although individuals with different continental origins can usually be separated by genome-wide SNP data, some level of

overlap exists between particular continental regions<sup>48</sup> (FIG. 2a,b). This genetic overlap increases with decreasing geographic distance, such as between regions within continents. For example, although a strong correlation between geographic and individual genetic distance exists within Europe, there is considerable overlap in genetic similarity distributions between neighbouring European subpopulations, which prevents completely accurate determination<sup>52</sup> (FIG. 2c). Nevertheless, genome-wide European population substructure, although small overall, seems to be large enough to make determining regional European ancestry with large numbers of autosomal SNPs practically feasible<sup>52</sup> (FIG. 2c). Although dense, whole-genome SNP microarrays are extremely powerful for the identification of biogeographic ancestry, they are not very useful in practice for analysis of forensic samples. Therefore, small sets of autosomal ancestry-informative or ancestry-sensitive DNA markers have been developed for DNA-based inference of biogeographic ancestry at the level of continental resolution<sup>53–56</sup> (BOX 4; FIG. 2b).

Overall, we expect that there will be many forensic cases where DNA-based inference of biogeographic ancestry will be useful for guiding police investigations to find unknown persons or victims (for an example of one such case, see REF. 57). However, when and how biogeographic ancestry DNA testing is applied to solve forensic questions must be determined by the level of geographic substructure detectable using available DNA marker sets. Current knowledge provides detection mainly at the levels of large geographic regions such as continents, although some subregional differentiation may be possible. It also needs to be kept in mind that, with some exceptions<sup>58</sup>, genetic diversity

**Intelligence-led policing**

A strategic, future-oriented and targeted approach to crime control, focusing upon the identification, analysis and ‘management’ of persisting and developing ‘problems’ or ‘risks’.

**Genetic admixture**

The process of mixing of two or more groups whose ancestors had been separated (usually long before).

**Genetic population substructure**

The absence of random mating within a population, leading to allele frequency differences among subpopulations.

**Ancestry-informative or ancestry-sensitive DNA markers**

DNA markers that show marked allele frequency differences between populations from different geographic regions, and are therefore useful for determining the probable biogeographic ancestry of an individual.

## Box 4 | DNA-based inference of biogeographic ancestry: an example

One of the authors (P.d.K.) requested a genetic ancestry test from two direct-to-consumer (DTC) genetic testing companies, 23andMe and deCODEme, out of the many that offer such tests<sup>30</sup> using genome-wide SNP data. In addition, he was also tested with 47 autosomal ancestry-sensitive markers (ASMs) developed by the authors<sup>54</sup>. We use this example to represent a forensic case involving a hitherto unknown suspect with the key question being the reconstruction of his most likely biogeographic ancestry. Notably, P.d.K. is of Dutch European origin (for at least five generations), and he is of European appearance. We recognize three different approaches of inferring biogeographic ancestry from DNA information (although the two companies do not provide exact details on which method they used to determine biogeographic ancestry estimates).

The first approach involves genetic similarity estimation and plotting procedures based on genetic polymorphisms that have been identified in reference samples from individuals of known geographic origins who have similar genetic profiles to the sample of interest. This approach uses methods such as principle component analysis<sup>147</sup>, Laplacian eigenvector<sup>148</sup> or multidimensional scaling analysis of genetic distance matrices (see FIG. 2 for examples of multidimensional scaling). Subsequently, the unknown sample is added to the analyses and its position is visualized and interpreted. Following this approach, both companies, using genome-wide SNP data, predicted a European ancestry that was further refined to northern European ancestry. Using autosomal ASMs, a European ancestry was concluded.

The second approach employs Bayesian cluster algorithms for genetic admixture estimation using software such as Structure<sup>149</sup>, Frappe<sup>150</sup> or Admixture<sup>151</sup>. The software estimates the relative admixture proportions of a given number of *a priori* defined ancestral groups contributing to the genome of each individual. Accepting and understanding many assumptions, one can first estimate the most optimal number of ancestral groups among all reference individuals<sup>48</sup>. Subsequently, one can apply a conditional test to estimate the admixture proportions in the unknown sample(s). According to this approach and using genome-wide SNP data, one company predicted a 100% European biogeographic ancestry and the other company predicted a 92% European, 6% East Asian, and 2% African ancestry. Autosomal ASMs gave a similar 91%, European, 3% East Asian, and 6% African ancestry estimate.

The third approach is to infer the paternal and maternal biogeographic ancestry separately using non-recombining Y chromosome and mitochondrial DNA (mtDNA). Although both companies use slightly different sets of SNPs, their predictions were the same. Both revealed haplogroup J (specifically J1c5a) for mtDNA, and haplogroup I2 (I2a2\*) for the Y chromosome. Interestingly, these haplogroups are not typical for an individual of northern European biogeographic ancestry, but are characteristic for individuals from eastern or south-eastern Europe, or the Middle East.

This case study demonstrates that even the use of different DNA marker sets, presumably different reference data sets, and different approaches of data analysis can lead to similar predictions of biogeographic ancestry on the regional (that is, continental) geographic level, which is reassuring. However, it also shows the extreme care with which one should interpret results when more detailed geographic conclusions are derived. Further issues of uncertainty, although not highlighted in this example, arise with biogeographic ancestry inference of people who are of mixed genetic ancestry involving ancestors of various geographic regions.

#### Principle component analysis

A multivariate analysis that provides a new coordinate system, the axes of which (the principal components) successively account for the maximum amount of variance and are uncorrelated with each other.

#### Laplacian eigenvector

An analysis which — compared with, for example, principal component analysis (PCA) — is a statistical tool one can use to achieve dimension reduction of highly complex sets of (genetic) data. It has a major advantage over PCA in that it compares each individual only with its close neighbours, rather than with all other individuals (here, closeness refers to genetic relatedness, not geographic distance).

#### Multidimensional scaling analysis

A dimensionality reduction technique, similar to principal component analysis, in which points in a high-dimensional space are projected into a lower-dimensional space while approximately preserving the distance between points.

#### Genetic distance matrices

A matrix of values expressing the degree of genetic differentiation between two or more populations (or individuals).

#### Bayesian cluster algorithms

A probabilistic technique for evaluating the grouping of individuals or populations. Hypotheses are evaluated by their posterior probabilities.

#### Relative admixture proportions

The relative contribution of two or more parental populations to a hybrid population.

#### Genome-wide association studies

Analysis across the genome using association models to identify regions that contribute to genetic variation in a phenotype. These studies typically analyse data from high-density SNP arrays.

rarely follows political borders. Not surprisingly, a recent plan of the UK Border Agency to apply DNA ancestry testing to investigate whether asylum-seekers come from Somalia or Kenya has caused an outcry among human geneticists<sup>59</sup>. Obviously, such a plan lacks any genetic basis. Furthermore, we discourage the use of DNA-based biogeographic ancestry information, which is usually performed with genetic markers not involved in appearance traits, to make statements about a person's EVCs. Such statements are likely to be error-prone as almost no appearance trait is restricted to a certain geographic region. In principle, appearance information should be estimated using markers from genes that are functionally involved in, or strongly associated with, particular EVCs (see next section). However, until such information becomes available on a more detailed level, continental biogeographic ancestry may be used carefully in some cases to get a general idea about a person's appearance, but this should only be applied to individuals whose ancestors come from a single continental region (as can be established with DNA-based ancestry testing). So far, there is no clear understanding how the extent of genetic admixture within an individual is reflected in his or her appearance traits.

*Estimating appearance traits from DNA.* Surprisingly little is known about the genetic factors that determine human appearance. The introduction of genome-wide association studies (GWA studies) for finding genes involved in complex traits provided information for some EVCs such as eye, hair and skin colour, pigmentation-related traits such as freckles<sup>60–65</sup>, stature (for the most recent study, see REF. 66), and hair morphology<sup>67–69</sup>. Furthermore, GWA studies for EVCs involved in disease traits such as nonsyndromic cleft lip with or without cleft palate (NSCL/P)<sup>70,71</sup>, autosomal-dominant woolly hair (ADWH)<sup>72</sup> and male baldness (androgenetic alopecia (AGA))<sup>73,74</sup> may provide genetic leads towards understanding normal appearance variation, although such traits are not directly useful for forensic applications.

Obviously, association is not prediction, and the genetic effect size of a SNP on a phenotype, the number of independently contributing SNPs, and non-genetic influences (for example, environmental impact) all determine how accurately a phenotype can be predicted from genotypes. Of all appearance traits studied so far, eye colour is the most accurately predictable. A systematic study<sup>75</sup> involving thousands of Europeans showed that a model including 15 SNPs predicted eye colour with prevalence-adjusted accuracies of 0.93 for

brown and 0.91 for blue eye colour as expressed by the area under the receiver characteristic operating curves (AUC), with 1 reflecting complete accuracy and 0.5 random prediction. This is significantly more accurate than an earlier attempt to predict eye colour from DNA<sup>60,75</sup>. Strikingly, one particular SNP (rs12913832 in *HERC2*) covers most of the DNA-predicted eye colour information, with AUC values of 0.899 for brown and 0.877 for blue<sup>75</sup>. From these findings, the IrisPlex system was developed for DNA-based eye colour prediction. This technology includes a highly sensitive multiplex genotyping assay for the six most predictive SNPs from six pigmentation genes, as well as a statistical model with an underlying database that allows reliable categorical eye colour prediction independent of biogeographic ancestry<sup>76</sup>. The IrisPlex assay has already been forensically validated<sup>77</sup>, and is therefore now available for forensic case work. FIGURE 3 illustrates the predictive power of IrisPlex for human eye colour prediction. Others<sup>78,79</sup> have also reported SNPs for eye colour prediction — most of which overlap with IrisPlex — but none of these reach similarly high levels of eye colour predictability.

One problem with applying DNA prediction of categorical appearance traits in general, and eye colour in particular, is the expected variation in conceptual understanding of categorical trait information. For instance, different people may assign the same eye to different colour categories, and may therefore look for different individuals when using an eye colour category provided from DNA prediction. To minimize this problem, a study investigated the genetic basis of quantitative variation in eye colour, and not only found three new eye colour genes, but also explained ~50% of quantitative eye colour variation using SNP data<sup>64</sup>. If developed further, such an approach may lead to the use of colour charts or printouts for eye colours predicted by DNA, which would avoid practical uncertainties.

Prediction analysis using SNPs has also been performed for hair colour, and most accurately for red hair<sup>60</sup>, which is largely determined by a single gene, *MC1R*. SNPs in *MC1R* provide strong predictions of red hair colour<sup>80,81</sup>, and the uses of such predictions for forensic applications were examined years ago<sup>82</sup>. A recent systematic study investigated 46 SNPs from 13 genes for their predictive value for different hair colours, and reported AUC values of 0.93, 0.87, 0.82 and 0.81 for red, black, brown and blond hair, respectively, based on 13 single or compound DNA markers from 11 genes<sup>83</sup>. In addition, this model enabled similar hair colour categories to be distinguished between, such as between red and blond-red, and between blond and dark-blond. The lowest prediction accuracy for blond hair may be the result of age-dependent hair colour change<sup>83</sup>. The design of a multiplex assay and its forensic validation is currently in progress (M.K., unpublished observations). An earlier study reported that three SNPs together explained 76% of total variation of hair melanin in a cross-population study<sup>78</sup>. Two of these have recently been confirmed to be highly predictive for hair colour<sup>83</sup>, whereas the third one seems to reflect biogeographic ancestry rather than hair colour<sup>83</sup>.

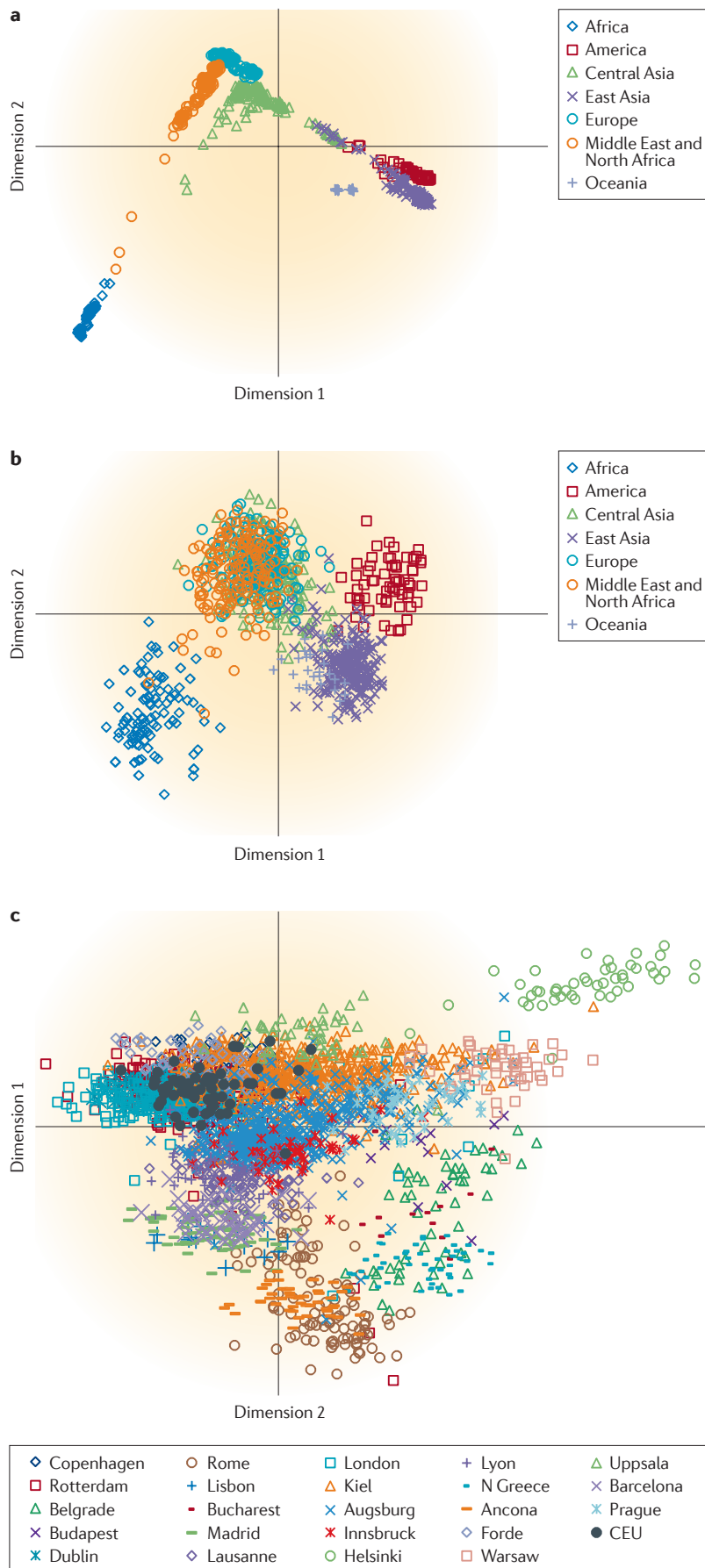
**Skin reflectance**  
Measurable light reflectance of the skin, which depends (among other things) on skin pigmentation.

**Figure 2 | Genetic substructure of human populations.** ▶ The extent of genetic population substructure allows DNA-based inferences of biogeographic ancestry at the level of worldwide continental resolution (a,b) and at the subregional level, such as for Europe (c). Two-dimensional plots are shown from multidimensional scaling analyses of pairwise identity-by-state distances. Distances in a and b were averaged across all markers on a worldwide scale with 938 samples from 53 populations from the Human Genome Diversity Project-Centre Etude Polymorphisme Humain. a | Plot derived using genome-wide SNPs (Illumina Human 650Y array, plot is based on 644,258 autosomal SNPs after quality control)<sup>47</sup>. b | Plot derived using 47 ancestry-sensitive SNPs<sup>54</sup> (also used to infer P.d.K.'s biogeographic ancestry, see BOX 4). c | A similar plot on a European scale, with 2,457 samples from 23 subpopulations using genome-wide SNPs (Affymetrix GeneChip Human Mapping 500K Array Set) and an additional 60 unrelated samples from Utah residents of assumed northern and western European ancestry (CEU; samples from Centre Etude Polymorphisme Humain) with matching SNP data from the International HapMap Project<sup>52</sup>. The plot is based on 253,572 autosomal SNPs after quality control and merging of data sets. The position of the CEU samples in this genetic map of Europe closely agrees with biogeographic ancestry expectation derived from family records regarding the European subregion.

A few GWA studies have been performed on human skin colour<sup>60,63</sup> and have highlighted several genes, but our knowledge regarding the genetic determination of skin colour variation is still incomplete. For instance, a recent study showed that three SNPs from three pigmentation genes explained only 46% of total variation in skin reflectance<sup>78</sup>. This value is significantly lower than the amount of variation in eye colour or hair melanin explained by SNPs in the same individuals<sup>78</sup>. One problem with using GWA studies for mapping skin colour genes is that the full trait variation cannot be covered. Considering samples from different continents in the same GWA study to cover worldwide variation in skin colour will highlight all SNPs with strong differences between continental regions, and will therefore deliver numerous false-positive hits for a particular skin colour. Hence, GWA studies for skin colour are carried out within continental groups, such as Europeans<sup>60</sup> and Asians<sup>63</sup>, but are limited as they can only cover a proportion of the total skin colour variation. By using an evolutionary genetic approach, five SNPs were identified that explain 82% of skin colour variation in worldwide populations<sup>84</sup>. These markers are expected to be useful for individual skin colour prediction.

SNPs have also been used for model-based prediction of human body height<sup>85</sup>. However, as may be expected from the small individual SNP effects on height (for example, 0.4 cm<sup>86</sup>), the accuracy of prediction achieved so far is very low (AUC of 0.65, where 0.5 reflects random prediction). Hence, many more height-associated SNPs are needed for DNA-based height prediction to be accurate enough for practical applications. A recent GWA study of more than 180,000 individuals identified





at least 180 loci that influenced adult height<sup>66</sup>, and has made numerous SNPs available for future testing of their predictive value.

Another trait that is externally visible to some extent and that could be valuable for predicting appearance is individual age. Two DNA-based approaches for age prediction based on age-dependant accumulation of mtDNA deletions and age-dependant telomere shrinkage have been suggested for forensic applications, but their practical value seems limited for various reasons<sup>87</sup>. Genome-wide studies on age-dependant changes of gene expression or DNA methylation patterns may provide leads for establishing more suitable age-predictive biomarkers<sup>88,89</sup>. The most promising currently known single DNA marker for individual age estimation from blood sources, however, is a particular somatic rearrangement in the T cell receptor locus, particularly its by-product the signal joint T cell receptor excision circles (sjTRECs)<sup>90</sup>. Building on previous knowledge that the number of T cells and therefore the number of sjTRECs declines with increasing age, this study showed that normalized sjTREC quantification can be used to estimate age from blood samples with a standard error of  $\pm 9$  years. It can also be used for categorical age prediction, with high AUC values from 0.89 to 0.97 for age categories separated by 20 years. The same study described a sensitive quantitative PCR test for sjTREC quantification, which does not seem to be affected by DNA degradation<sup>90</sup>, although careful forensic validation testing remains to be done.







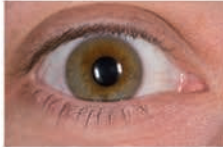
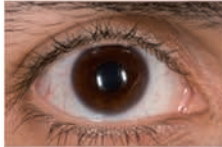







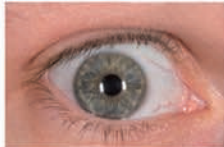

Clearly, any test to estimate EVCs from DNA will only be able to recover the genetic component of a trait; hence, any environmental impact on the EVC variation will in principle contribute to the uncertainty of DNA-based appearance prediction. Furthermore, all currently DNA-predictable EVCs are group-specific. Although group-specific traits are expected to be useful to reduce a large group of potential suspects, it is the individual-specific appearance prediction that is sought. In this respect, it will be crucial to consider facial morphology. The human face is largely determined by genetic components, as the strong facial resemblance of monozygotic twins demonstrates. However, the genetic factors that determine facial morphology are not yet known.

**Linking sample donors with criminal acts**

Information that supports a link between sample donors who have been identified by DNA profiling and actual criminal acts is crucial, but until recently attempts to extract such data directly from crime scene samples have been limited. We consider at least two important aspects for which advances in human genomics and molecular biology have provided advances: determining the sample's cellular origin and estimating the sample's age and deposition time.

**Determining cellular origin.** Current methods used in forensics for determining the cellular source of a forensic sample, also called forensic tissue identification, are mainly presumptive: that is, they cannot tell us with

# REVIEWS

 <b>Bl: 0.97</b> Int: 0.02 Br: 0.01	 <b>Bl: 0.94</b> Int: 0.04 Br: 0.02	 <b>Bl: 0.87</b> Int: 0.08 Br: 0.05	 Bl: 0.13 Int: 0.18 <b>Br: 0.69</b>
 <b>Bl: 0.96</b> Int: 0.03 Br: 0.01	 <b>Bl: 0.94</b> Int: 0.04 Br: 0.02	 <b>Bl: 0.87</b> Int: 0.08 Br: 0.05	 Bl: 0.09 Int: 0.15 <b>Br: 0.76</b>
 <b>Bl: 0.96</b> Int: 0.03 Br: 0.01	 <b>Bl: 0.90</b> Int: 0.07 Br: 0.03	 Bl: 0.34 <b>Int: 0.49</b> Br: 0.17	 Bl: 0.09 Int: 0.15 <b>Br: 0.76</b>
 <b>Bl: 0.96</b> Int: 0.03 Br: 0.01	 <b>Bl: 0.90</b> Int: 0.07 Br: 0.03	 Bl: 0.13 Int: 0.18 <b>Br: 0.69</b>	 Bl: 0.01 Int: 0.06 <b>Br: 0.93</b>
 <b>Bl: 0.95</b> Int: 0.03 Br: 0.02	 <b>Bl: 0.90</b> Int: 0.07 Br: 0.03	 <b>Bl: 0.42</b> Int: 0.22 Br: 0.36	 Bl: 0.00 Int: 0.04 <b>Br: 0.96</b>
 <b>Bl: 0.95</b> Int: 0.03 Br: 0.02	 <b>Bl: 0.90</b> Int: 0.07 Br: 0.03	 Bl: 0.30 Int: 0.25 <b>Br: 0.45</b>	 Bl: 0.00 Int: 0.03 <b>Br: 0.97</b>
 <b>Bl: 0.95</b> Int: 0.03 Br: 0.02	 <b>Bl: 0.90</b> Int: 0.07 Br: 0.03	 Bl: 0.31 Int: 0.14 <b>Br: 0.55</b>	 Bl: 0.00 Int: 0.03 <b>Br: 0.97</b>
 <b>Bl: 0.94</b> Int: 0.05 Br: 0.01	 <b>Bl: 0.87</b> Int: 0.08 Br: 0.05	 Bl: 0.31 Int: 0.14 <b>Br: 0.55</b>	 Bl: 0.00 Int: 0.01 <b>Br: 0.99</b>
 <b>Bl: 0.94</b> Int: 0.04 Br: 0.02	 <b>Bl: 0.87</b> Int: 0.08 Br: 0.05	 Bl: 0.23 Int: 0.13 <b>Br: 0.64</b>	 Bl: 0.00 Int: 0.01 <b>Br: 0.99</b>
 <b>Bl: 0.94</b> Int: 0.04 Br: 0.02	 <b>Bl: 0.87</b> Int: 0.08 Br: 0.05	 Bl: 0.23 Int: 0.13 <b>Br: 0.64</b>	 Bl: 0.00 Int: 0.01 <b>Br: 0.99</b>

◀ **Figure 3 | DNA-based prediction of human eye colour.** Eye photographs of 40 individuals, ordered according to DNA-based eye colour prediction probabilities as derived from the IrisPlex system; eye colour phenotypes were not considered in the ordering of the photographs. The order starts with the highest probability for blue in the upper left corner and with the highest probability for brown in the lower right corner. Prediction probability estimates for blue (Bl), brown (Br) and intermediate (Int) eye colour as derived from the IrisPlex system are provided with each eye picture<sup>76</sup>. For only three of the 40 individuals (highlighted by a red box) IrisPlex eye colour prediction appears to be non-informative or inaccurate relative to visual inspection of eye colour; for two of these persons none of the three prediction probabilities is informative, and for one IrisPlex prediction reveals a different colour from visual inspection. This illustrative example demonstrates the power of DNA-based eye colour prediction in general and of the IrisPlex system in particular. Reproduced, with permission, from REF. 76 © (2010) Elsevier.

certainty that a sample is from a particular body source, because positive test results are also possible from other sources<sup>91</sup>. However, recent developments indicate that this situation might be improved. A study that applied genome-wide expression microarrays to blood and saliva samples, stored for up to 180 days, revealed sample separation strictly according to body fluids<sup>92</sup>. Subsequent RT-PCR confirmation delivered various suitable mRNA markers for the determination of blood and saliva, respectively<sup>92</sup>. Currently, several mRNA-based multiplex RT-PCR assays are available for the parallel determination of venous blood, saliva, semen and menstrual blood<sup>93–96</sup>. The value of proposed mRNA markers for vaginal secretion is controversial<sup>94,97,98</sup>, but may be improved by applying microbial markers<sup>99</sup>. A recent study identified several mRNA markers and provided highly sensitive RT-PCR methods for skin identification in forensic applications<sup>100</sup>. Although some of the mRNA markers proposed for forensic body fluid determination were tested successfully in samples that were up to 16 years old<sup>95,101,102</sup>, additional environmental conditions such as humidity and temperature are expected to influence mRNA stability, and remain to be tested rigorously.

As an alternative, microRNA (miRNA) markers have started to be explored for potential use in body fluid determination<sup>103,104</sup>. A clear advantage of miRNAs over mRNAs is their small size, 18–22 bp, which makes them likely to have higher *in vitro* stability than mRNAs. One study, which applied genome-wide microarrays covering more than 700 human miRNAs to samples from all forensically relevant body fluids, revealed a clear separation of individual samples according to body fluids<sup>104</sup> (FIG. 4). Subsequent RT-PCR testing of the microarray candidates revealed several suitable miRNA markers for blood and semen, respectively, but none for saliva, menstrual blood or vaginal secretions<sup>104</sup>. Although an earlier study suggested miRNA markers for differentiating all body fluids<sup>103</sup>, the results for saliva, menstrual blood and vaginal secretions have yet to be confirmed<sup>104</sup>, and warrant additional investigation. One potential problem for their forensic application is that some (but not all) miRNAs are not human-specific, owing to strong evolutionary conservation<sup>104</sup>. This issue is not seen with mRNA, particularly when TaqMan RT-PCR is applied.

#### DNA methylation

A DNA modification in which a methyl group is added to cytosine. Methylation inhibits gene expression and is maintained through DNA replication and cell division.

#### TaqMan RT-PCR

A proprietary system (developed by Applied Biosystems) that allows the progression of a PCR reaction to be monitored in real time.

Recently, the finding that blood, semen, saliva and skin samples carry distinguishable DNA methylation profiles has led to the concept of DNA methylation-based forensic tissue identification<sup>105</sup>. Forensic validation is necessary to judge the practical use of this new approach, which appears promising.

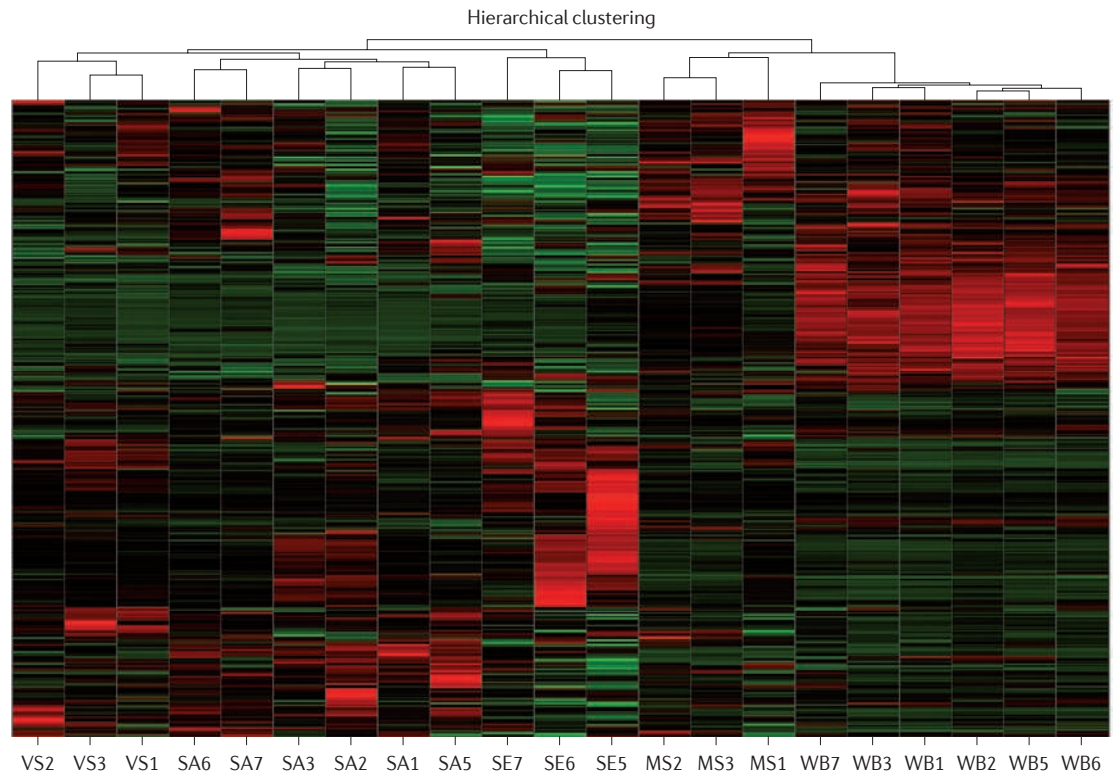
**Timing sample placement.** Two distinct aspects of timing are relevant in the context of crime scene investigation. The first addresses time in terms of calendar-date — that is, how many days, weeks or months ago a sample was deposited at a scene. One might expect that such timing could be determined if the *in vitro* degradation of biological materials is time-dependant and understood well-enough for practical applications. Along these lines, several approaches based on different aspects of differential RNA degradation were recently developed<sup>106–108</sup>. However, more systematic studies of time-dependant degradation of biomarkers (including RNA) need to be carried out to evaluate additional environmental factors that are expected to influence biodegradation, such as temperature and humidity.

The second time-related aspect is the time of day or night at which a sample was deposited at a scene, thereby bringing a chronobiological aspect into forensic analysis. Recently, a proof-of-principle study<sup>109</sup> demonstrated that the well-known 24 hour concentration profiles of two circadian hormones, melatonin and cortisol, can be extracted reliably from small blood samples while controlling for the amount of starting material. This approach offers time estimations in the order of about 4–5 hours from fresh and aged blood stains. It is not without caveats — for example, night-time melatonin biosynthesis is suppressible by exposure to light<sup>109</sup> — but such molecular systems hold promise for the accurate estimation of sample deposition time. For this to be achieved, more circadian biomarkers that have different peak times during day and night and have signals independent from external sources will need to be identified.

#### Future perspectives

The advances we have discussed suggest that forensic investigations will improve substantially in the near future. However, several challenges remain. One example is the obvious need for methods to identify multiple donors of a mixed sample, especially when the donors are of the same gender. Here, the use of next-generation DNA sequencing technologies from single molecules<sup>110</sup>, especially those not involving PCR<sup>111</sup>, is expected to bring progress. These technologies also provide possibilities for analysing small amounts of degraded DNA, which is important for many forensic cases and for DVI.

In terms of FDP, further research is needed to determine the level of detail at which biogeographic ancestry and appearance information can be inferred from biological materials. In addition, the ability to estimate individual-specific appearance via DNA-predicted facial morphology is an important goal towards identifying unknown persons. If such prediction turns out to be possible, it is unlikely to be achievable with small



**Figure 4 | Differentiation of forensically relevant body fluids using genome-wide microRNA expression data.** Unsupervised hierarchical clustering of genome-wide expression data from various forensically relevant human body fluids of several individuals using Exiqon LNA™-modified oligonucleotide microarrays, which cover 718 micro RNAs (miRNAs), in semen (SE), saliva (SA), vaginal secretion (VS), menstrual blood (MS) and whole venous blood (WB). Plot is based on 458 miRNAs expressed above threshold<sup>104</sup>. All samples are clustered according to their body fluid source, illustrating the potential of miRNAs to be used in cellular origin identification. Modified, with permission, from REF. 104 © (2010) Springer.

sets of genetic markers. Therefore, new technologies are needed for the parallel genotyping of large numbers of SNPs, which can successfully deal with small amounts of degraded DNA.

Finally, another important aspect is a more detailed reconstruction of crime events with biomarkers. Current expectations include determining the time

of sample donation and sample age in much greater detail than has hitherto been possible. However, these capabilities may go much further, such as estimating the genetic predispositions of perpetrators for forensically relevant behavioural traits<sup>112</sup> and the physiological conditions of perpetrators and victims at the time of criminal acts.

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**Competing interests statement**

The authors declare no competing financial interests.

**FURTHER INFORMATION**

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 23andMe: <https://www.23andme.com>  
 Centre d'Etude du Polymorphisme Humain (CEPH)  
 Database: <http://www.cephb.fr/en/cephdb/>  
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