State-of-the-Art and Future Prospects of Canine STR-Based Genotyping

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Abstract: The dog is the most common domestic animal in human environments and in many situations a dog may be a victim, a perpetrator or a link between a suspect and a crime scene. Therefore, biological material derived from dogs may constitute evidence in forensic caseworks and it may be necessary or helpful to obtain genetic profiles that would aid individual identification. Currently, the assessment of the genetic diversity of breeds, population structure, inbreeding, and the development of methodologies for population assignment are important areas of research in dogs and related species such as the grey wolf. Therefore, canine short tandem repeat (STR)-based genotyping is used by a significant number of population geneticists; however, for reasons we present here, it is utilized by a relatively small number of forensic practitioners. An extensive bibliographic search revealed a highly fragmented canine genotyping community working under less than well defined standards. In this work, we discuss the present developments and limitations of STR-based canine genotyping. Furthermore, we recommend that a collaborative strategy for the implementation of standardization and harmonization is crucial to the development of forensic canine genotyping.

Keywords: *Canis lupus familiaris*, non-human genotyping, short tandem repeat.

BRIEF HISTORY

The dog (*Canis lupus familiaris*) is widely recognized as the most common pet in North American and European households. Despite the constant presence of dogs in human environments, canine derived evidence is not frequently analyzed in forensic caseworks and, consequently, is seldom reported in the literature [1-9]. Numerous situations may occur in which canine genetic identification constitutes an important or even the only source of evidence. Animals can be victims of cruelty and theft; therefore the identification of the remains of a lost or a stolen dog may have to be performed. Also, animals can be perpetrators of a crime, and it may be necessary to identify an animal involved in an attack on a person or other animal. Furthermore, an unrestrained animal may cause an accident or be responsible for property damage. Moreover, animals can also be regarded as "silent witnesses": the analysis of animal DNA transferred as hair, saliva, blood, urine, or feces may provide a link between a suspect to a crime scene or to a victim. Requests of individual profiles, identification for dog paternity investigation and breed registries are growing in demand, as shown by the increasing number of commercial laboratories worldwide that now offer those services.

The development of polymerase chain reaction (PCR) in the late 1980's facilitated the analysis of polymorphic sequences ubiquitously distributed throughout genomes; particularly, tandem repeated units of one to five bp known as short tandem repeats (STR) or microsatellites were able to be analyzed. STR are highly utilized in forensics, population genetics, molecular ecology and related areas because of their relatively straightforward analysis, especially due to the widespread use of capillary electrophoresis. Human populations have been extensively surveyed *via* STR-based methodologies over the last 15 years, particularly after the establishment of validated STR panels and databases such as the CODIS (Combined DNA Index System, www.fbi.gov). Isolation and description of canine STRs began in the early 1990's [10, 11] and the physical and linkage mapping of the dog genome have become a major source of mapped loci (e. g. [12-18]). Francisco and colleagues pioneered the mapping of a set of highly polymorphic tetranucleotide loci in 1996; however, other studies mostly reported the mapping of dinucleotide loci. In 1994 Zajc and colleagues [19] first proposed a method of paternity testing in dogs based on microsatellite sequences, followed by similar works in subsequent years (e. g. [20-25]). These early studies attempted to obtain insights into population genetics and phylogenetic relationships between extant and extinct canine populations using STRs originally isolated from the dog genome. The list of investigated species included the Ethiopian wolf [26], the red wolf [27], the red fox [28], the Arctic fox [29] and the grey wolf [30].

Canine genotyping has been particularly performed in non-forensic fields in order to characterize the genetic structure and the diversity within and among dog breeds and wolf populations (e. g. [31-43]). The majority of these studies were motivated by conservation concerns and tried to assess population structure, genetic diversity and inbreeding in endangered populations or small breeds (e. g. [30, 44-58]). As

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natural populations become increasingly threatened by species introduction and the relative abundance of sympatric species, several authors have also discussed the potential hybridization among closely related species, such as the grey wolf, the red wolf, the coyote, and the dog [59-67]. Population assignment based on individual genotypes is becoming an interesting challenge, with a number of researchers using their data to estimate the probability of an individual belonging to a given reference breed or group (e. g. [42, 53, 56, 58, 63, 68-74]). In the literature, two other interesting examples of canine genotyping were mentioned: the establishment of the clonal origin and evolution of a canine transmissible venereal cancer [75, 76], and the analysis of a putative dog clone as an independent test to determine the validity of cloned cell lines [77].

DNA quantification is an important step in STR-based analysis prior to conducting PCR, especially in less than pristine sample. This issue was addressed by Evans and colleagues [78], who proposed a real-time PCR assay targeting the canine-specific coding region of the Melanocortin-1 receptor (*MC1R*) gene. They report the successful incorporated of this method into routine analysis of canine biological material.

A DIFFICULT COMING OF AGE

As presented in the previous section, canine STR-based genotyping has been performed in a wide range of studies for at least 15 years. In order to assess the use of STR markers, we surveyed the literature reporting canine genotyping data between 1996 and 2009 (72 publications) regardless of the objectives of the work (e.g. forensic and non-forensic), from which references to a total of 345 loci were compiled.

A highly dispersed use of STR markers was strikingly evident, as the majority (56%) were used in a single study, 36% in 2 to 6 studies, and only 8% (29 loci) were used in 7 to 27 studies (Fig. **1**). Furthermore, a high proportion (35%) of the most commonly used 29 markers (Table **1**) consisted of dimeric loci. This type of marker is known to have significant germinal and somatic instability, and generates a high amount of stuttering products that are difficult to interpret [79]. For these reasons, dimeric STRs are absent from the international panels for human identification [80], whereas tetrameric loci that have negligible slippage and easy resolution of consecutive alleles are the repeat type of election.

Moreover, it was observed in the literature that genotyping results are generally published in a non-standardized manner due to the lack of a repeat-based nomenclature of alleles (and sometimes even a consistent nomenclature of loci). Recently, a small number of studies [6, 81-84] have discussed this issue and fully characterized some markers as a pre-requisite for routine applications. These studies represent individual in-house efforts to produce suitable panels of

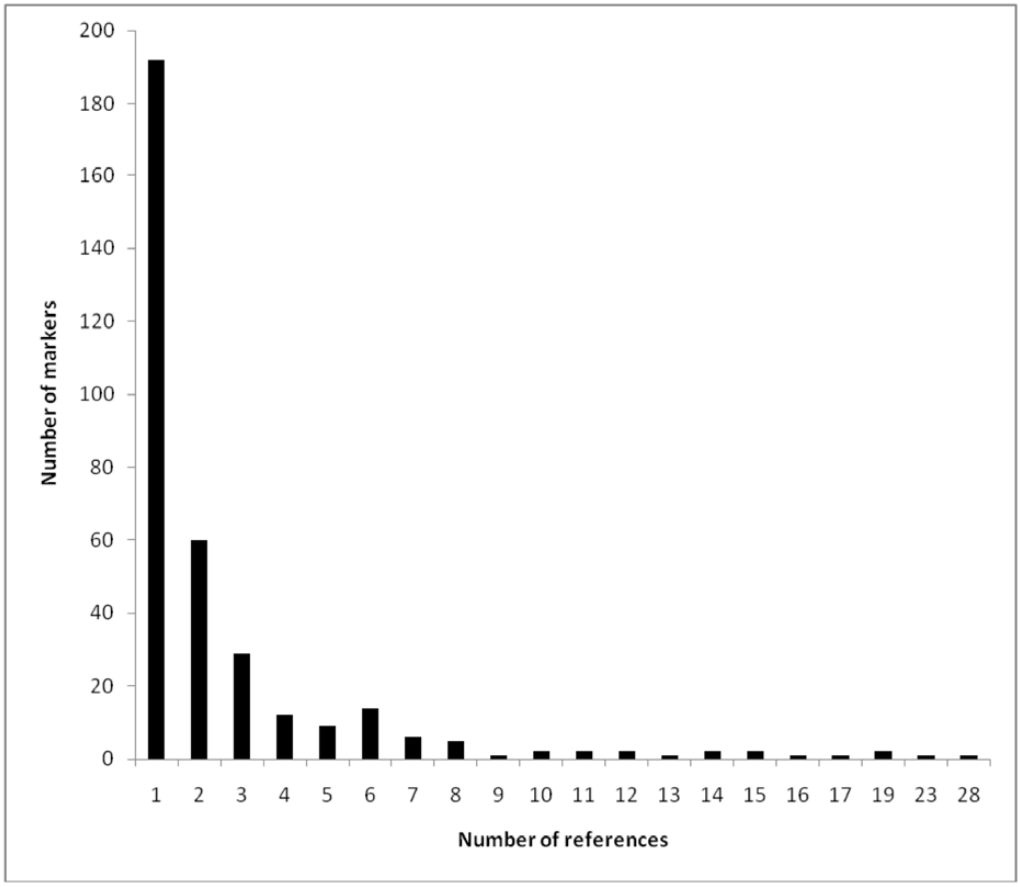

Fig. (1). Number of canine STR markers referenced in published studies. The distribution is based on a total of 345 loci referred in a total of 72 publications.

Table 1. List of Commonly Used Markers Referred in 7 to 28 Studies Compiled from a Bibliographic Survey Including 72 Publications. Repeat Type, and Availability in at Least One Study of Allele Frequencies and Estimates of Forensic Parameters

Marker	Type of Repeat	Allele Frequencies	Forensic Parameters	References
C09.250	Dimeric	YES	NO	[34, 36, 37, 49, 52, 58, 60, 61]
CPH02	Dimeric	YES	NO	[23, 34, 36, 37, 49, 51, 52, 60, 61, 71]
CPH03	Dimeric	YES	NO	[34-37, 52, 60, 61, 63, 71]
CPH04	Dimeric	YES	NO	[49, 51, 52, 59-61, 63, 71]
CPH07	Dimeric	YES	NO	[49, 52, 59-61, 63, 71]
CPH08	Dimeric	YES	NO	[23, 34-37, 49, 51, 52, 60, 61]
CXX.109/u109	Dimeric	YES	NO	[30, 40, 43, 48, 51, 58, 66, 72]
CXX.213/u213	Dimeric	YES	NO	[30, 49, 51, 52, 60-62, 66]
CXX.225/u225	Dimeric	YES	NO	[30, 40, 43, 45, 48, 51, 58, 64, 66, 72]
u250	Dimeric	YES	NO	[30, 40, 48, 51, 62, 66, 70, 72]
FH2001	Tetrameric	YES	YES	[22, 34-37, 43, 46, 51, 54, 57, 58, 62, 67, 68, 70, 71, 73]
FH2004*	Tetrameric	YES	YES	[22, 23, 33, 34, 36, 37, 46, 48, 57, 63, 67, 68, 73, 74, 81]
FH2010*	Tetrameric	YES	YES	[3, 4, 6, 7, 9, 23-25, 43, 48, 50, 52, 57, 58, 60-62, 67, 70, 71, 73, 74, 81, 83, 84]
FH2017	Tetrameric	YES	YES	[40, 43, 48, 62, 63, 70, 73]
FH2054*	Tetrameric	YES	YES	[3, 4, 6, 7, 9, 22-25, 33-37, 41-43, 46, 48, 50, 51, 57, 58, 62, 64, 67, 68, 70, 71, 83, 84]
FH2079*	Tetrameric	YES	YES	[3, 6, 7, 9, 24, 25, 34, 48-50, 60-62, 67, 83, 84]
FH2088	Tetrameric	YES	YES	[22, 33, 43, 46, 48, 49, 51, 52, 54, 57, 60-62, 67, 68, 70, 73]
FH2096	Tetrameric	YES	NO	[43, 48, 49, 51, 52, 54, 60-62, 67, 70, 72]
FH2132*	Tetrameric	YES	YES	[9, 22, 35, 46, 68, 71, 83, 84]
FH2137	Tetrameric	YES	YES	[22, 34, 35, 46, 57, 68, 71]
PEZ01/CATA1	Tetrameric	YES	YES	[3, 4, 6, 7, 24, 25, 43, 48, 50, 51, 62, 70]
PEZ03*	Tetrameric	YES	YES	[3, 6, 7, 24, 25, 34, 38, 43, 48, 51, 62, 72, 82]
PEZ05	Tetrameric	YES	YES	[3, 4, 6, 7, 24, 25, 34, 40, 43, 48, 50, 51, 57, 62, 70, 72, 73]
PEZ06*	Tetrameric	YES	YES	[3, 6, 7, 9, 24, 25, 48, 50, 62, 83, 84]
PEZ08*	Tetrameric	YES	YES	[3, 6, 7, 24, 25, 34, 36, 37, 43, 50, 57, 58, 62, 70, 82]
PEZ12*	Tetrameric	YES	YES	[3, 6, 7, 9, 24, 25, 34, 38, 40, 43, 48, 50, 57, 62, 83, 84]
PEZ15*	Tetrameric	YES	YES	[7, 9, 24, 25, 50, 57, 83, 84]
PEZ20*	Tetrameric	YES	YES	[3, 4, 6, 7, 24, 25, 48, 50, 62]
VWF.X*	Hexameric	YES	YES	[9, 30, 43, 48, 51, 52, 60-62, 70, 72, 73, 83, 84]

*Markers characterized with respect to sequence structure (see also Table **2**).

markers for identity and kinship analysis, and have proposed repeat-based nomenclatures for a total of 29 loci (Table **2**). In the majority of cases, these descriptions were accompanied by estimates of forensic parameters for identification and parentage testing based on population data. Nevertheless, data comparison remains generally difficult, if not impossible, because most publications are prior to the studies that presented a standardized repeat-based nomenclature of alleles.

Table 2. List of Canine STR Loci Characterized at Sequence Level with a Published Repeat-Based Allele Nomenclature. Type of Repeat, Availability of Allele Frequencies and Estimation of Forensic Parameters, and Works where these Markers were used are also Indicated

Marker	Type of Repeat	Marker Characterization Reference	Allele Frequencies	Forensic Parameters	Other Publications Referring the Characterized Marker
C38	Tetrameric	[81]	YES	YES	[74]*
FH2004	Tetrameric	[81]	YES	YES	[22, 23, 33, 34, 36, 37, 46, 57, 63, 68 73, 74]*
FH2010	Tetrameric	[81, 84]	YES	YES	[3, 6, 23-25, 48, 50, 52, 57, 58, 43, 60-62, 70, 71, 73, 74]*
FH2054	Tetrameric	[84]	YES	YES	[3, 6, 22-25, 33-37, 46, 48, 50, 51, 57, 58, 62, 64, 68, 70 42, 43, 71, 83]
FH2079	Tetrameric	[84]	YES	YES	[3, 6, 24, 25, 34, 48-50, 52, 62 43, 60, 61, 63, 70, 83]
FH2087Ua	Tetrameric	[84]	YES	YES	[83]*
FH2087Ub	Tetrameric	[84]	YES	YES	[83]*
FH2132	Tetrameric	[84]	YES	YES	[22, 35, 46, 68, 71 83]*
FH2161	Tetrameric	[82]	NO	NO	[34, 63, 71]
FH2328	Tetrameric	[82]	NO	NO	[34, 73]
FH2361	Tetrameric	[81]	YES	YES	[34, 73, 74]*
FH2611	Tetrameric	[84]	YES	YES	[57, 58 83]*
FH2658	Tetrameric	[81]	YES	YES	[57, 74]*
FH3210	Tetrameric	[81]	YES	YES	[74]*
FH3241	Tetrameric	[81]	YES	YES	[74]*
FH4012	Tetrameric	[81]	YES	YES	[74]*
PEZ02	Tetrameric	[84]	YES	YES	[34, 36, 37, 73 83]*
PEZ03	Tetrameric	[82]	NO	NO	[3, 6, 24, 25, 34, 38, 43, 48, 51, 62, 72]
PEZ06	Tetrameric	[82, 84]	YES	YES	[3, 6, 24, 25, 48, 50, 57, 62, 83]*
PEZ08	Tetrameric	[82]	NO	NO	[3, 6, 24, 25, 34, 36, 37, 43, 50, 57, 58, 62, 70]
PEZ10	Tetrameric	[82]	NO	NO	[24, 25, 34, 50]
PEZ12	Tetrameric	[84]	YES	YES	[3, 6, 24, 25, 34, 38, 40, 48, 50, 57 62, 83*43]
PEZ15	Tetrameric	[84]	YES	YES	[24, 25, 50, 57 83]*
PEZ20	Tetrameric	[6]	YES	YES	[3, 24, 25, 48, 50, 62]
REN214L11	Tetrameric	[81]	YES	YES	[74]*
VWF.X	Hexameric	[84]	YES	YES	[30, 40, 48, 51, 52, 60-62, 70, 72, 73, 83*43]
Wilms-T	Tetrameric	[84]	YES	YES	[34, 83]*
ZUBECA4	Tetrameric	[84]	YES	YES	[83]*
ZUBECA6	Tetrameric	[84]	YES	YES	[83]*

*Studies that reported genotyping results according to the loci's proposed nomenclature.

The extremely low number of fully characterized loci in common use (FH2004, FH2010, FH2054, FH2079, FH2132, PEZ03, PEZ06, PEZ08, PEZ12, PEZ15, PEZ20 and VWF.X) may be explained by the fact that most researchers performing canine genotyping are not forensic practitioners. Therefore, these researchers are not under pressure to comply with forensic standards or to contribute to universal databases that allow for inter-laboratory comparisons. Also, and in contrast with the commercial availability of validated human genotyping systems, commercial canine genotyping systems are, at present, inexistent. Despite the value that an STR multiplex kit would bring to the canine genotyping community (forensic and non-forensic), a single commercial kit (Stockmarks® for Dogs Canine Genotyping Kit, Applied Biosystems, Foster City, CA) that was briefly available was subsequently discontinued in 2005. This kit did not include an allelic ladder nor was a nomenclature of the included markers ever published; therefore it is not surprising that the commercial success of the product was greatly compromised from the very beginning. A more convenient product has not become commercially available, further promoting the production of internal laboratory solutions. Although this activity is healthy by principle and has originated useful tools, we have most likely reached a point where greater benefits can only be achieved through broader collaborations.

FUTURE PROSPECTS

At present, the use of canine STRs is highly fragmented among laboratories, and lacks standardization and harmonization. This is probably the most important reason for the limited use of STR analysis derived from canine evidence in forensic caseworks. Regrettably, we must agree that the forensic laboratories that are used to perform high standard identification and kinship analysis in humans have justifiable reasons to be reluctant. Forensic laboratories rely on validated STR panels, great amounts of comparable population data and well-defined statistical aids to interpret of the results of human analysis, and are therefore generally unwilling to venture onto shakier grounds.

Recently, Kanthaswamy and colleagues [73] selected 18 markers from existing panels in order to assemble a standardized and validated [85] canine forensics panel that will be commercialized in the future by Finnzymes Oy (Espo, Finland) under the designation Canine 2.1 STR Multiplex Reagent Kit. They also have established a database of canine STR genotypes in an updatable format to allow for the inclusion of new data submitted by laboratories that will use this panel. If it proves to be adequate, this kit may constitute an practical tool for the laboratories that wish to implement canine STR analysis.

Regardless of the species under examination, STRs constitute a powerful tool that can be used for identity and kinship testing. The biological principles and the theories, methodologies and technologies are well established for human testing and may only require slight adaptations for non-human testing. Basically, a proficient forensic practitioner is equally competent to analyze either human or animal biological evidence. Potentially motivated by the asymmetry of the standards for human genotyping when compared to non-human genotyping, Budowle and colleagues, in 2005 [86], proposed the first set of guidelines to inspire quality practices that withstand legal scrutiny for animal genetic identity testing was proposed by in a condensed yet detailed report that is well worth consulting.

Two decades of collective experience in human STR-based genotyping has enabled us to identify the primary issues for the efficient development of non-human genotyping systems. Specifically, it is essential to determine which markers should be used and how to apply them. For the sake of simplicity, the main criteria to consider when selecting suitable STR markers for forensic genotyping may be summarized in the following points: absence of genetic linkage, tetrameric loci (preferably in perfect repeat structures), high level of informativeness, low occurrence of mutations and null alleles, specificity and absence of stutter peaks, and balanced PCR amplification in multiplex reactions. The following points summarize actions necessary for the development of forensic canine genotyping: selection of core STR loci for canine identity testing and establishment of multiplexed panels, development of an internationally recognized allele nomenclature based on the number of repeats determined by sequencing of frequent alleles [87], implementation of sequenced allelic ladders, validation of STR panels for forensic analysis, establishment of publicly available databases, collaborative efforts for the collection of population data, and proficiency and quality control testing (e. g. [88]).

In conclusion, the literature showed a relatively intense use of canine STR markers in the conservation, ecology, phylogeny and forensic fields in the last 15 years; however, inter-laboratory variability was considerable, in contrast with the high level of harmonization and standardization attained in a comparable period of human STR analysis. An integrated community where forensic and non-forensic researchers may converge needs to be established in order to further develop methodologies. In this perspective, it would be highly advantageous that major international societies such as the International Society of Animal Genetics (www.isag.org.uk) and the International Society of Forensic Genetics (www.isfg.org) collaborate in the development of guidelines for animal genotyping and specifically canine genotyping. These actions would certainly contribute to the emergence of a forensic community performing non-human analysis and the discussion of the particular challenges of a pioneering area.

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