TECHNICAL NOTE

Takashi Yoshimoto,[1] B.S.; Toshimichi Yamamoto,[1] Ph.D.; Rieko Uchihi,[1] Ph.D.; Keiji Tamaki,[2] M.D.; Xiu-Lin Huang,[3] M.D.; Masaki Mizutani,[1] M.D.; Miwa Tanaka,[1] B.D.S.; John A.L. Armour,[4] B.M., B.Ch.; and Yoshinao Katsumata,[1] M.D.

A New Triplex STR System Without Irregular Alleles by Silver Staining and its Potential Application to Forensic Analysis*

REFERENCE: Yoshimoto T, Yamamoto T, Uchichi R, Tamaki K, Huang X-L, Mizutani M, et al. A new triplex STR system without irregular alleles by silver staining and its potential application to forensic analysis. J Forensic Sci 2001;46(3):448–452.

ABSTRACT: In order to increase the discriminating power of DNA analysis in forensic science, we devised a new triplex STR system using three novel STR loci we previously reported, D14S299 (wg1c5), D15S233 (wg1d1), and 9q2h2. We designated this system a CDH triplex system. The CDH triplex system showed a high discriminating power, especially in Caucasians. This system is composed of three STR loci showing only regular tetranucleotide repeat alleles. We easily enlarged the databases mainly of Japanese, using this system, and compared them with those of Caucasian and Chinese. This CDH triplex system therefore appears to be useful for forensic practice.

KEYWORDS: forensic science, DNA typing, short tandem repeat, population genetics, triplex, polymerase chain reaction, D14S299, D15S233, 9q2h2

Short tandem repeat (STR) loci are composed of tandemly repeated sequences that can be two to seven base pairs in length. Some kits based on simultaneous analysis of multiple STR loci (multiplex) have been validated for forensic application. The combined use of two or three kits allows us to type more than ten STR loci. One such system composed of 13 STR loci showed an average match probability rarer than one in a trillion (1). This system also showed an average exclusion probability of 99.99% with the complete data available for mother, child, and the alleged father in parentage analysis (1).

However, the apparent possibility of germ line mutations in such STR systems is not negligible (2) for parentage analysis. When a single exclusion case in paternity testing is encountered, it is desirable to type other STR loci to ascertain whether the mutation is genuine or not.

We have already reported the individual properties of three novel STR loci, D14S299 (wg1c5) (3), D15S233 (wg1d1) (3), and 9q2h2 (4). They seem to be especially useful for forensic analysis because of their regular tetranucleotide repeat alleles at all loci in spite of their comparatively high heterozygosities. Therefore, they can be typed easily by silver staining on denaturing polyacrylamide gels.

"In this study, we devised a new triplex STR system, and designated the CDH triplex system from a part of each clone name, wg1"c"5, wg1"d"1, and 9q2"h"2. This system applies those STR loci for forensic analysis, especially further analysis of single exclusion cases in parentage testing. Using this triplex STR system, we improved the Japanese databases for the three STR loci, and compared them with those of Caucasians and Chinese.

Materials and Methods

Sample Preparation

Whole blood samples were collected from unrelated Japanese (Nagoya area) individuals, and DNA was extracted as previously described (5). Some stored DNA samples of Chinese (Peking area) and Caucasians (UK) were also used. Venous blood samples were also collected from a paternity trio (mother, child, and alleged father), and DNA was extracted as well.

PCR Amplification

PCR typing of the three STR loci, wg1c5, wg1d1, and 9q2h2, was performed simultaneously in 10 μL of a buffer (6) containing 1 μM of the primers for wg1c5 (3) and 9q2h2 (4), 0.5 μM of the primers for wg1d1 (3), 0.5 U *Taq* DNA polymerase (Perkin Elmer, NJ), and 10 ng template DNA. After an initial denaturation at 95°C for 3 min, the following PCR cycle was repeated for 30 cycles: de-

[1] Research student, associate professor, assistant professor, postgraduate students, and professor, respectively, Department of Legal Medicine and Bioethics, Postgraduate School of Medicine, Nagoya University, 65 Tsurumacho, Showa-ku, Nagoya 466-8550, Japan.

[2] Professor, Department of Legal Medicine, Sapporo Medical University School of Medicine, Chuoku, Sapporo, 060-8556, Japan.

[3] Postdoctoral fellow, Department of Legal Medicine, Tokai University School of Medicine, Bohseidai Isehara 259-1193, Japan.

[4] Reader, Department of Genetics, School of Clinical Laboratory Sciences, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH, UK.

* Supported in part by Grants in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

Received 8 Oct. 1999; and in revised form 28 March 2000, 20 June 2000; accepted 22 June 2000.

naturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Final extension was at 72°C for 10 min. Each primer sequence are shown as follows; wg1c5a: 5′-GATCTCAATAAACATTGATACTGG-3′, wg1c5b: 5′-CTGCATGAGCTAAAGCATACTG-3′, wg1d1a: 5′-AGGTCTGGGTGACAGAACA-3′, wg1d1b: 5′-CACCACGTCCCGCCTCTAAT-3′ 9q2h2a: 5′-ACAGAGCAAGACTCTGTCAC-3′, 9q2h2b: 5′-GTATACACTTGAGCTAGTAGC-3′.

Purification of Single Alleles and Sequencing

Purification, cloning and sequencing of single alleles of the present three loci were performed as described previously (4,7,8).

Preparation of Ladder Cocktail

Plasmids including each single allele were analyzed on an ABI 373XL (PE Applied Biosystems, CA) automated sequencer. The products of each locus were diluted, mixed together, reanalyzed, and balanced to produce single ladders which were them mixed together to form a cocktail in proportions so that all densities were balanced on the stained gel.

Typing

PCR products were separated by electrophoresis on 6% denaturing polyacrylamide gels (8 M urea), followed by silver staining (9). Alleles were determined by comparison with the allelic ladder marker described above.

Statistical Analysis

Tests for the Hardy-Weinberg equilibrium (HWE) were carried out using the homozygosity test (10), likelihood ratio test (11), and exact test (12). The probabilities of the likelihood ratio test and the exact test were estimated based on 10 000 shuffling experiments. Each allele frequency at the three loci was pairwise compared between each population using the Chi-square test.

Results and Discussion

Nomenclature and Properties at Each Locus

The nomenclature of alleles at the present three loci follows the recommendations of the DNA commission of the ISFH (13,14) in which the number of complete tandem repeats observed is designated by digit(s), and some sequence data has already been published (4,7,8). An electrophoretic pattern of the present CDH triplex system with the allelic ladder markers in a parentage testing was shown in Fig 1. We could genotype all samples at each locus compared to the allelic ladders certainty. The properties, such as chromosome location, common sequence motif, and size range at each locus were arranged in Table 1. Although the common sequence motif of D14S299 seemed to be highly complex, surprisingly, we have observed only regular tetranucleotide alleles at least in hominoid in our phylogenetic study (in preparation).

Enlargement of the Databases and Statistical Properties

Databases were easily enlarged using the present triplex system, and the revised ones are shown in Table 2. The expected heterozygosities (12) were almost the same as those reported previously. At the loci of wg1c5 and wg1d1, the expected heterozygosity of the Japanese population was the lowest, while that at the locus of 9q2h2 was almost the same as those in the other two populations.

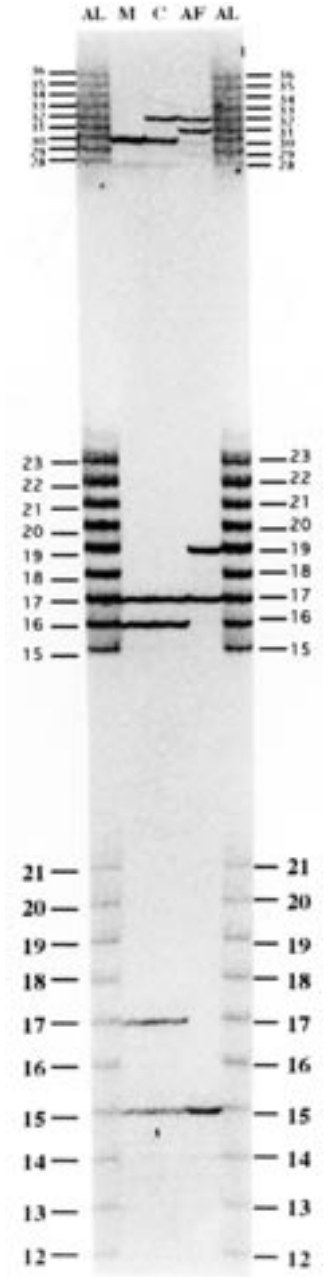

FIG. 1—*An electrophoretic pattern of the present CDH triplex system in a parentage testing. AL: allelic ladder markers; M: mother; C: child; AF: alleged father.*

Each database at three loci in Japanese was easily enlarged using the present triplex system by increasing 120 individuals to former databases (4,7,8) as shown in Table 2. Allele frequencies at 9q2h2 in Chinese, and at D15S233 and 9q2h2 in Caucasian were newly calculated (15). No significant deviations from Hardy-Weiberg equilibrium were observed using the homozygosity test, likelihood ratio test, and exact test (Table 3).

The statistical properties, such as the observed heterozygosity (Obs. Hz), expected heterozygosity (Exp. Hz, 16), power of discrimination (PD, 17), and polymorphism information content (PIC, 18), were calculated as shown in Table 4. Both Hz at D15S233 in all populations were highest in three loci. Both Hz of Caucasian at

TABLE 1—*Properties of the three STR loci in the present triplex STR system.*

Locus Designation	Chromosome Location	Common Sequence Motif	Size Range (bp)
D14S299	14	$(GGAT)_n$ AGAT $(GGAT)_2$ GGAA AAAT GGAT AGAT GGAT GGGT $(GGAT)_{0,2}$ GAAT $(GGAT)_n$ GAAT ATAT GGGT $(GGAT)_2$ $(GGGT)_{0-1}$ $(GGAA)_n$	299–343
D15S233	15	$(AGGA)_n$ $(GGGA)_n$	195–227
9q2h2	2p	$(GGAA)_n$	131–167

TABLE 2—*Allele frequency distributions at the three STR loci, D14S299, D15S233 and 9q2h2 in three different populations.*

	D14S299 (wglc5)				D15S233 (wgld1)				9q2h2		
Allele	Japanese* (n = 328)	Chinese (n = 113)	Caucasian (n = 112)	Allele	Japanese* (n = 356)	Chinese (n = 93)	Caucasian* (n = 112)	Allele	Japanese* (n = 274)	Chinese* (n = 115)	Caucasian* (n = 111)
28	0.005	0.004	0	13	0	0	0.009	12	0.002	0.004	0.005
29	0.084	0.071	0.049	14	0	0	0.013	13	0.055	0.057	0.063
30	0.453	0.407	0.246	15	0.028	0.048	0.121	14	0.184	0.226	0.171
31	0.229	0.265	0.254	16	0.173	0.134	0.210	15	0.323	0.348	0.293
32	0.130	0.119	0.201	17	0.317	0.328	0.241	16	0.292	0.239	0.306
33	0.038	0.044	0.063	18	0.118	0.145	0.063	17	0.091	0.070	0.108
34	0.043	0.049	0.134	19	0.291	0.253	0.210	18	0.029	0.039	0.050
35	0.005	0.009	0.045	20	0.053	0.051	0.076	19	0.015	0.017	0.005
36	0.011	0.027	0.009	21	0.014	0.011	0.054	20	0.005	0	0
37	0	0	0	22	0.003	0	0.004	21	0.004	0	0
38	0.003	0	0	23	0.003	0					
39	0.002	0	0								
40	0	0.004	0								

* Present study.

TABLE 3—*Tests for Hardy-Weinberg equilibrium.*

	Japanese			Chinese			Caucasian		
Locus	D14S299	D15S233	9q2h2	D14S299	D15S233	9q2h2	D14S299	D15S233	9q2h2
Homozygosity test	0.5382	0.6046	0.8024	0.7259	0.5217	0.1306	0.3871	0.2516	0.7745
Likelihood Radio test	0.6150	0.2892	0.8779	0.0813	0.8483	0.1607	0.6019	0.5501	0.6187
Exact test	0.7191	0.2122	0.9429	0.1714	0.9216	0.0802	0.6007	0.7311	0.6111

TABLE 4—*Statical properties at three loci in the three populations.*

	Japanese			Chinese			Caucasian		
Locus	D14S299	D15S233	9q2h2	D14S299	D15S233	9q2h2	D14S299	D15S233	912h2
Obs. Hz*	0.729	0.778	0.770	0.752	0.807	0.704	0.839	0.866	0.784
Exp. Hz†	0.717	0.768	0.765	0.743	0.785	0.764	0.812	0.830	0.777
PD‡	0.882	0.904	0.908	0.892	0.920	0.901	0.927	0.942	0.904
PIC§	0.680	0.731	0.728	0.704	0.749	0.724	0.781	0.804	0.739

* Observed heterozygosity.
† Expected heterozygosity (16).
‡ Power of discrimination (17).
§ Polymorphism information content (18).

TABLE 5—*Comparison of probability of paternity exclusion (PE) values at the present three STR loci among three populations.*

Locus	Japanese	Chinese	Caucasian
D14S299	0.5000	0.5283	0.6222
D15S233	0.5540	0.5782	0.6562
9q2h2	0.5502	0.5456	0.5639
Combined	0.8997	0.9096	0.9434

all three loci were higher than those of other populations. All PD values were more than 0.88, and the combined PD with this triplex was calculated at 0.9990, 0.9991, and 0.9996 in Japanese, Chinese, and Caucasian, respectively.

We also calculated the probability of paternity exclusion (PE, 19) values at each locus and the combined PE value of three loci in three populations (Table 5). Each value at all loci and the combined PE value of Caucasian was the highest in them. These values were similar to those of other loci in a nineplex kit (20).

Pairwise Comparison of Allele Distributions Among Three Populations

We compared pairs of populations using the revised databases, and found that there was no significant difference in allele distribution between Caucasians and either of the two Asian populations at the locus of 9q2h2 (Table 6). Allele distributions usually show highly significant difference between such very different populations as Caucasians and Asians at ordinary STR loci like the present two, wg1c5 and wg1d1. The simple allele structure of 9q2h2 (Table 1) may be related to the apparent stability of allele distribution, although further study is needed to verify this.

Mutation Rates

We could not calculate each mutation rate at all loci because not enough trio samples confirmed their parenthood; but we have not observed any mutations of these STRs among a few decades of our trio samples. However, Brinkmann et al. (21) reported that the mutation rate of repeat regions in STRs showed a positive correlation to the geometric mean of the number of variable uninterrupted repeats, and that this relation can be expressed as an exponential function. Accordingly, we have estimated the mutation rates at D14S299, D15S233, and 9q2h2 as approximately 0.2, 0.2, and 0.7% from the geometric mean of the number of variable uninterrupted repeats, 11.0, 11.9, and 15.3, respectively. The rates of the former two loci were similar to D21S11 (0.180%) and vWA (0.199%), and that of the latter locus was similar to ACTBP2 (0.684%). This rate (0.7%) was relatively high because the sequence structure was very simple and the number of repeats was large. However, since this value exceeded what was logically expected, the actual one may be smaller.

TABLE 6—*Pairwise comparison among the three populations at each present STR locus.*

Population Comparison	Locus	$\chi 2$	P-value
Japanese-Chinese	D14S299	5.153	0.5153
	D15S233	7.400	0.2854
	9q2h2	5.121	0.5283
Japanese-Caucasian	D14S299	57.46	1.46×10^{-10}
	D15S233	64.29	6.01×10^{-12}
	9q2h2	6.528	0.3611
Chinese-Caucasian	D14S299	29.79	4.30×10^{-5}
	D15S233	29.09	5.84×10^{-5}
	9q2h2	8.599	0.1974

Usefulness of the Present Triplex STR System

When we tried to amplify DNA extracted from a one-year-old bloodstain by the present system, we could correctly type all loci even though from 0.5 ng of template DNA without any allele drop out.

We designated the present STR system the CDH triplex system. Since all three loci have only regular tetranucleotide repeat alleles, each with high discriminating power, and have nonoverlapping allele size ranges, this system can accurately type their alleles by electrophoresis on a denaturing polyacrylamide gel followed by silver staining in which the initial cost performance is lower than the fluorescent format. This system would therefore seem to be useful as a supplementary tool in forensic practice, especially for forensic scientists unavailable in any fluorescent formats, and it will be more useful if a new fluorescent multiplex system including these three STR loci will be constructed in future.

References

1. Chakraborty R, Stivers DN, Su B, Zhong Y, Budowle B. The utility of short tandem repeat loci beyond human identification: Implications for development of new DNA typing systems. Electrophoresis 1999;20: 1682–96.
2. Thomson JA, Pilotti V, Stevens P, Ayres KL, Debenham PG. Validation of short tandem repeat analysis for the investigation of cases of disputed paternity. Forensic Sci Int 1999;100:1–6.
3. Armour JAL, Neumann R, Gobert S, Jeffrey AJ. Isolation of human simple repeat loci by hybridization selection. Hum Mol Genet 1994;3:599–605.
4. Mizutani M, Huang X-L, Tamaki K, Yoshimoto T, Uchihi R, Yamomoto T, et al. Evaluation of two new STR loci 9q2h2 and wg3f12 in a Japanese population. Legal Med 1999;1:25–8.
5. Tamaki K, Yamamoto T, Uchihi R, Katsumata Y, Kondo K, Mizuno S, et al. Frequency of HLA-DQA1 alleles in the Japanese. Hum Hered 1991;41:209–14.
6. Jeffreys AJ, Neumann R, Wilson V. Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. Cell 1990;60:473–85.
7. Kojima T, Yamamoto T, Yoshimoto T, Tamaki K, Huang X-L, Ohtaki H, et al. Tetrameric short tandem repeat (STR) system D15S233 (wg1d1): Sequencing and frequency data in the Japanese and Chinese population. Legal Med 1999;3:119–26.
8. Yoshimoto T, Tamaki K, Katsumata S, Huang X-L, Uchihi R, Tanaka M, et al. Sequence analysis of alleles at a microsatellite locus D14S299 (wg1c5) and population genetic comparisons. Int J Legal Med 1999; 113:15–8.
9. Goldman D, Merril CR. Silver staining of DNA in polyacrylamide gels, linearity and effect of fragment size. Electrophoresis 1982;3:24–6.
10. Weir BS. Independence of VNTR alleles defined by fixed bins. Genetics 1992;130:873–87.
11. Chakraborty R, Pornage M, Guegue R, Boerwincle E. Population genetics of hypervariable loci: Analysis of PCR based VNTR polymorphisms within population, In: Burke T, Dolf G, Jeffreys AJ, Wolf R, editors. DNA fingerprinting: Approaches and applications, Birkhauser Verlag, Berlin, 1991;127–43.
12. Guo SW, Thompson EA. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. Biometrics 1992;48:361–72.
13. DNA Commission of the International Society for Forensic Haemogenetics. Recommendations of the DNA Commission of the International Society for Forensic Haemogenetics relating to the use of PCR-based polymorphisms. Forensic Sci Int 1992;55:1–3.
14. DNA recommendations—1994 report concerning further recommendations of the DNA commission of the IFSH regarding PCR-based polymorphisms in STR (short tandem repeat) systems. Int J Legal Med 1994;107:159–60.
15. http://www.med.nagoya-u.ac.jp/legal/jalm/al_index.hhtml.

16. Nei M. Estimation of average heterozygosity and genetic distance from small number of individuals. Genetics 1978;89:583–90.
17. Grunbaum BW, Selvin S, Pace N, Black DM. Frequency distribution and discrimination probability of twelve protein genetic variants in human blood as functions of race, sex, and age. J Forensic Sci 1978;23:577–87.
18. Botstein D, White RL, Scolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 1980;32:314–31.
19. Chakraborty R, Stivers DN. Paternity exclusion by DNA markers: effect of paternal mutations. J Forensic Sci 1996;41:671–7.
20. Yamamoto T, Uchihi R, Nozawa H, Huang X-L, Leong Y-K, Tanaka M, et al. Allele distribution at nine STR Loci-D3S1358, vWA, FGA, TH01, TPOX, CSF1PO, D5S818, D13S317 and D7S820-in the Japanese population by multiplex PCR and capillary electrophoresis. J Forensic Sci 1999;44:167–70.
21. Brinkmann B, Klintschar M, Neuhuber F, Huhne J, Rolf B. Mutation rate in human microsatellites: influence of the structure and length of the tandem repeat. Am J Hum Genet 1998;62:1408–15.

Additional information and reprint requests:
Toshimichi Yamamoto, Ph.D.
Department of Legal Medicine and Bioethics
Postgraduate School of Medicine
Nagoya University
65 Tsuruma-cho, Showa-ku,
Nagoya 466-8550,
Japan