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ORIGINAL ARTICLE

Reduced-volume and low-volume typing of Y-chromosomal SNPs to obtain Finnish Y-chromosomal compound haplotypes

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Abstract Single-nucleotide extension is a widespread method for typing Y-chromosomal single-nucleotide polymorphisms. In our study, we validated a multiplex minisequencing assay in a reduced-volume and in a low-volume approach. A four-plex assay was performed in a 6-μL multiplex reaction in 96-well microtiter reaction plates, which can be directly used for capillary electrophoresis. In a second approach, a six-plex assay was performed on a chemically structured glass slide. Both techniques have proven to be highly sensitive as well as time- and cost-saving, which makes them a valuable option not only for forensic purposes but also for population genetic studies where large sample numbers have to be analyzed. In the present paper, both techniques are compared and applied to analyze a population sample from the area of Turku, Finland. The most common haplogroup was found to be N1c*, which is nearly absent in western and central European populations. Additionally, 11 short tandem repeat markers were analyzed to further discriminate Y-chromosomal lineages.

Marielle Heinrich and Tina Braun contributed equally to this work.

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Introduction

Single-nucleotide polymorphisms (SNPs) have proven to contain valuable information for identification [e.g., 1, 2] and also for population genetic purposes [e.g., 3]. Recently, Y-chromosomal [e.g., 4] as well as mitochondrial [5], autosomal [6], and X-chromosomal SNPs [7] were subjects of investigations.

Beside the analysis of population-specific SNP-defined haplogroups, the search for a suitable typing strategy for SNPs is a central point of research [e.g., 6, 8, 9]. Due to the fact that, in population genetic studies, a large number of samples usually have to be analyzed, an ideal typing strategy has to be convenient in handling as well as cost-efficient and time-saving. Additionally, the method should be highly sensitive to enable multiple typing of various markers from limited amounts of sample. As described previously, an enhancement of polymerase chain reaction (PCR) sensitivity can be achieved by the reduction of the total PCR reaction volume [10, 11]. A low-volume (LV) PCR performed in a 1-μL reaction on the surface of a chemically structured glass slide also presents enormous cost efficiency due to the reduction of PCR reagents [10].

The aim of the present study was to perform a multiplex single-nucleotide primer extension assay in a reduced-volume (RV) PCR of 6 μL and in a LV PCR of only1 μL and to assess the respective advantages. Thus, we developed a RV and a LV multiplex assay for PCR and minisequencing of Y-chromosomal SNPs. For validation, cell line DNA as well as a Finnish population sample was analyzed. The Finnish population is extraordinarily inter-

esting because it has an exceptional position among the Scandinavian populations. Most of the people in Finland speak a Finno-Ugric language, which has different origins than the languages spoken in the neighboring countries such as Sweden and Norway [12]. Additionally, several autosomal recessive diseases, which occur rarely in other European countries, occur relatively frequently in the Finnish population and vice versa [13]. Mitochondrial [14] and autosomal [15] DNA reveals the genetic homogeneity of the Finns, whereas Y-chromosomal lineages are notably reduced and point to a population substructure [16]. In this study, a Finnish population sample was used for the validation of a new technique for Y-chromosomal haplogroup assignment. To further discriminate Y-chromosomal lineages, 11 short tandem repeat (STR) markers were typed in addition to the binary polymorphisms [17] to obtain compound haplotypes (HTs).

Materials and methods

Samples

Blood samples from 84 as far as we know unrelated Finnish males were obtained during routine autopsies in the Department of Forensic Medicine at the University of Turku. DNA was extracted using phenol/chloroform or the Qiagen Blood Kit (Qiagen, Hilden, Germany). Quantification of the extracts was performed using the Human Quantifiler Kit (Applied Biosystems, Darmstadt, Germany).

RV and LV typing of Y-chromosomal SNPs

A total of nine Y-chromosomal SNPs (Fig. 1) were typed in two multiplex assays using different reaction platforms with the marker Tat (M46) included in both reactions to check for possible inconsistencies. Sensitivity of both assays was estimated by amplifying cell line DNA (Qantifiler Y standard DNA, Applied Biosystems) in twofold serial dilutions of 800 to 3 pg.

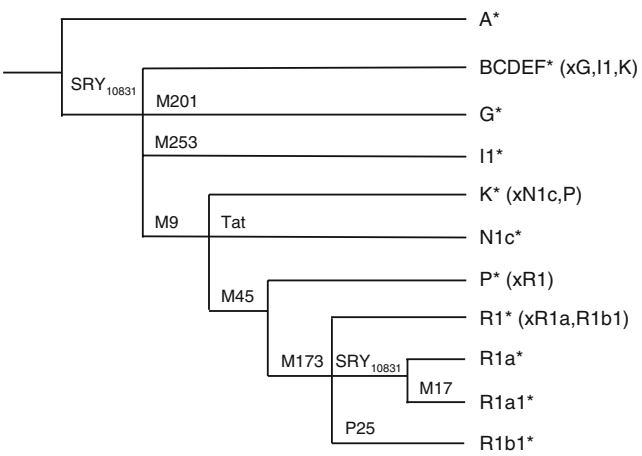

Fig. 1 Condensed phylogenetic tree of the Y chromosome according to the YCC [20] depicting the binary polymorphisms analyzed in this study

Four-plex assay (96-well format)

Four Y-chromosomal SNPs (Tat, M253, SRY10831, and M9) were amplified in a total reaction volume of 6 µL containing 0.6 µL 10x AmpliTaq Gold PCR buffer (containing 15 mM $MgCl_2$; Applied Biosystems), 0.5 µL dNTPs (2.5 mM each), 0.2 µL bovine serum albumin (20 mg/mL), 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 0.1 to 2 ng of genomic DNA. For primer concentrations and sequences, see Supplementary Table 1 of the Electronic supplementary material. Positive and negative controls were applied on each plate. PCR was performed in 96-well plates using a PTC gradient Mastercycler with an initial heating step at 95°C for 10 min; three cycles of 96°C for 30 s, 60°C for 30 s, and 65°C for 30 s; three cycles of 95°C for 30 s, 58°C for 30 s, and 65°C for 30 s; 30 cycles of 95°C for 30 s, 56°C for 30 s, and 65°C for 30 s; and a final extension at 65°C for 6 min. Of the PCR products, 3 µL was removed to enable an agarose gel electrophoresis or a second minisequencing reaction.

After amplification, each reaction was treated with exonuclease I and shrimp alkaline phosphatase by adding 1.2 µL ExoSAP-IT (USB Europe, Staufen, Germany) into each well of the reaction plate. The plate was incubated at 36°C for 60 min and at 80°C for 15 min.

For the minisequencing reaction, 1 µL of a master mix consisting of 0.7 µL SNaPshot reaction mix (Applied Biosystems) and 0.04–0.1 µM of each sequencing primer (see Supplementary Table 1 of the Electronic supplementary material for details) was placed into each well yielding a final reaction volume of 5.2 µL. The thermal cycling protocol was 25 cycles of 96°C for 10 s, 55°C for 55 s, and 60°C for 30 s.

Finally, minisequencing reactions were treated with 2 U of shrimp alkaline phosphatase (SAP, Roche, Mannheim, Germany) for 60 min at 37°C for dephosphorylation and 15 min at 75°C for inactivation of the enzyme.

Capillary electrophoresis was used for the separation and detection of the sequencing products, 20 µL HiDi formamide (Applied Biosystems) and 1 µL LIZ 120 internal size standard were added to each well, and the plate was mounted on a 3100 *Avant* Genetic Analyzer with Foundation Data Collection software v. 2.0 and Gene Mapper ID software v. 3.2 (Applied Biosystems).

Six-plex assay (slide format)

Amplification of six Y-chromosomal SNPs (M45, Tat, P25, M201, M17, and M173) was performed on chemically structured glass slides (AmpliGrid 480F, Olympus Advalytix, Munich, Germany) each with 48 reaction compartments (anchor spots) using an Eppendorf Mastercycler with in situ adapter (Eppendorf, Hamburg, Germany). Of a master mix, 1 μL containing 0.1 μL 10× PCR buffer, 0.1 μL dNTP mix (2.5 mM each), 1 μM each primer, and 0.5 U AmpliTaq Gold DNA polymerase were applied to each of the anchor spots, dried for 20 min at room temperature, resuspended by adding 1 μL DNA sample, and covered with mineral oil (Covering Solution, Olympus Advalytix). Positive and negative controls were applied on each slide. Thermal cycling proceeded as described.

Samples were treated with ExoSAP-IT by pipetting 0.4 μL of the enzyme mix onto each anchor spot and penetrating the oil cover with the pipette.

Minisequencing reaction was performed using 0.4 μL SNaPshot reaction mix (Applied Biosystems) and sequencing primers as indicated in Table 1. Reagents were transferred onto each reaction spot by pipetting through the oil cover. Thermal cycling was performed as described.

After minisequencing, each reaction was transferred into a PCR reaction tube and treated with 1 U SAP (Roche) prior to the analysis of the minisequencing products on a 3100 *Avant* Genetic Analyzer (Applied Biosystems).

Typing of Y-chromosomal STRs

In addition to the Y-chromosomal SNPs, 11 loci Y-STR HTs (DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, diallelic DYS385 [17], DYS437, DYS438, and DYS439) were analyzed using PowerPlex Y (Promega, Mannheim, Germany) in a 12.5-μL reaction volume. Further settings were as recommended by the manufacturer.

Intrapopulation diversity indices were calculated using Arlequin v. 3.1 software [18].

Table 1 Y-chromosomal haplogroups in a Finnish population sample from the area of Turku as revealed by RV and LV typing of Y-SNPs

HG	Frequency (n=86)
I1*	0.291 (25)
K*	0.035 (3)
N1c*	0.523 (45)
P*	0.012 (1)
R1*	0.023 (2)
R1a1*	0.093 (8)
n.a.	0.023 (2)

Haplogroup (HG) designation according to [20]. With the chosen set of markers, distinct haplogroups were assigned to all but two samples *n.a.* not assigned

Results and discussion

Typing strategy

Two new methods for RV and LV SNP typing based on the principle of single-nucleotide primer extension were developed. A four-plex RV assay in 96-well reaction plates and a six-plex LV assay on the surface of chemically structured chips were used for typing 86 unrelated males from Turku in the south–west of Finland. Typing of Y-chromosomal SNPs with these assays was successful for all samples analyzed, and there were no inconsistent results of the Tat typing run on both assays. Both reactions were validated and optimized with respect to the amounts of primer and template as well as buffer conditions for multiplex PCR and minisequencing. The protocols given above are the results of these optimization steps. It was possible to reduce the amount of SNaPshot reaction mix (Applied Biosystems) to 0.7 μL (RV) and 0.4 μL (LV) per reaction, respectively, instead of 5 μL as recommended by the manufacturer. This means enormous savings in reagents and costs.

For the six-plex assay, 48 reactions per glass slide were performed in a total PCR volume of 1 μL each and a final volume of 2 μL after minisequencing. For final treatment with SAP, the samples were removed from the anchor spots and transferred into reaction tubes. For the four-plex RV assay in a 96-well format, all reaction steps, from PCR to detection of minisequencing products, were performed in the same well. Thus, the RV assay is more convenient in handling and easily allows automation.

Both techniques described showed a high sensitivity with reproducible full allelic profiles down to 50 pg for both assays. Partial profiles could still be obtained from 12.5 pg of cell line DNA (Fig. 2). The method of minisequencing itself is ideally suited for the chemically structured chip as typing results are unambiguous; the difficulties typically observed with STR analysis do not occur with single-nucleotide extension [10]. However, batch-specific differences in the slide quality have been observed.

Even though the slide constitutes an “open” platform on which the single anchor spots were covered with mineral oil only, no cross-contamination was observed. These findings confirm previous observations made with the slide [10, 11]. However, performance of the LV assay is counterbalanced by its more complex processing, which includes a transfer of samples into reaction tubes prior to SAP treatment and

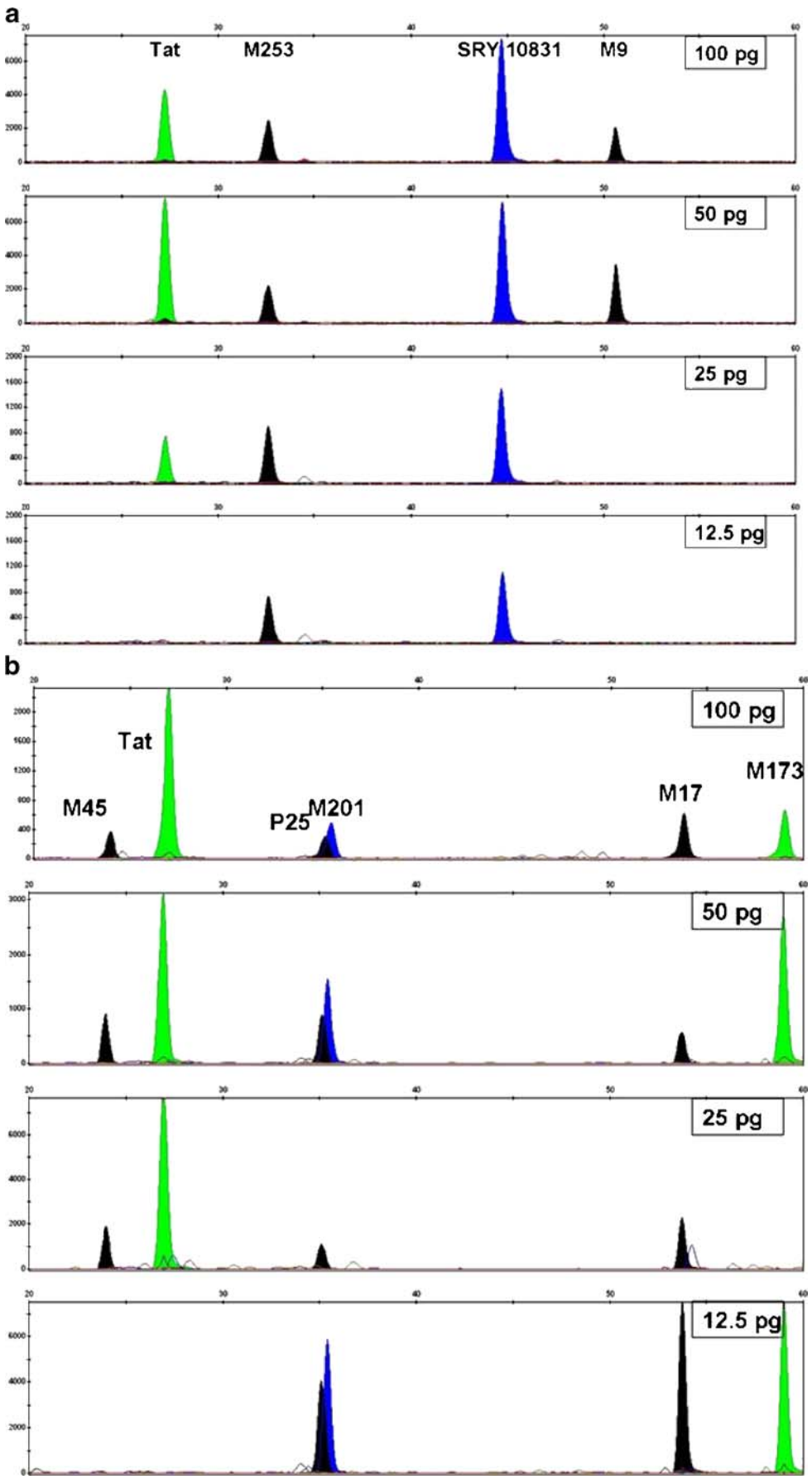

Fig. 2 Analysis of cell line DNA in twofold serial dilutions (100–12.5 pg shown) with the four-plex RV assay performed in 6 µL reactions in a 96-well plate (**a**) and the six-plex LV assay performed in 1 µL reactions on the glass slide (**b**). In both assays, 50 pg yielded full allelic profiles, while even with 12.5 pg of template DNA, partial profiles were obtained

detection. Nevertheless, the LV assay was found to be easy in handling, and even pipetting through the mineral oil cover is quite convenient. With both techniques, the time-consuming labeling, opening, and closing of the reaction tubes is reduced to a minimum or is even dispensable (RV assay).

As stated, the two techniques described comprise different advantages. A major disadvantage of the LV technique is the missing possibility to perform a second minisequencing reaction—or any other downstream application—from the same PCR product. This impossibility to double-check a PCR product might also be a critical point in forensic routine. When using a 6-μL reaction in the 96-well plate, 3 μL of the PCR product were removed after amplification and could be used, for example, for another minisequencing reaction or an agarose gel electrophoresis to check the amplification success before any further treatment. Moreover, expenses for 96-well plates are far lower than those for the chemically structured glass slides.

Altogether, the RV and LV assays allow robust and sensitive analysis of a high number of samples with relatively short hands-on time, which is useful not only in the forensic laboratory, but also whenever large sample numbers have to be analyzed, e.g., in population genetic studies.

Distribution of Y-chromosomal compound haplotypes in Turku, Finland

The described typing techniques were applied to a Finnish population sample from the area of Turku situated in the south–west of Finland. The SNP markers for both assays were chosen to span the main paragroups of the Y-chromosomal phylogenetic tree [19, 20] without resolving clades B* to F*, but with a higher resolution in paragroup P* which is the most common clade in many European populations [21–23], and M253, a very common mutation within the Finnish population [24] (cf. Fig. 1 for details).

With the two multiplex assays validated, it was possible to assign a Y-SNP haplogroup or clade to 98% of the samples (84 out of 86), cf. Table 1. In two samples, the haplogroup was not assigned because SRY10832 “G” and the ancestral state in all other binary markers were detected. Haplogroups N1c* [20] (formerly N3* [19]) and I1* [20] (formerly I1a* [19]) accounted for approximately 81 % of analyzed Y chromosomes (52.3% and 29.1%, respectively, cf. Table 1) leading to a comparatively low haplogroup diversity of 0.6208±0.0394. The haplogroup distribution detected in this study is more often found in the eastern than the western parts of Finland [25–27] but it still matches the findings of Lappalainen et al. for the province of south-western Finland [25]. The abundance of haplogroup N1c*(N3*) is typical for the northern and eastern European populations and demonstrates significant eastern contribution to the pool of Finnish Y chromosomes [28]. Haplogroups within the paragroup P*, which are the most common haplogroups in central and western European populations [21–23], were found in 11 samples only.

In addition to the binary Y-SNP markers, 11 highly polymorphic STR markers were analyzed. In 86 samples, a total of 62 different HTs were observed. Among them, 49 nonrecurring HTs were found, and the most common HT was present in eight individuals (cf. Supplementary Table 2 of the Electronic supplementary material). The HT diversity was found to be relatively high (0.9860±0.0057) for a Finnish population sample [29]. However, western provinces have shown higher Y-chromosomal HT diversity than the middle and eastern parts [29], and it is still lower than in other Scandinavian [30] or in other European populations [31]. Analysis of a greater number of Y-STR loci has been shown to significantly increase the HT diversity and to lower the level of interregional differentiation in the Finnish population; nevertheless, discrimination power is relatively low [32]. The most common HT found in the Turku sample is abundant in the Uralic–Yukaghir metapopulation according to the Y chromosome haplotype reference database (YHRD) [33] and is also observed predominantly in the north and east of Europe, i.e., Norway, Sweden (Swedish Saami [30]), Estonia, Lithuania, Belarus, and the Russian Federation, but to a much lesser extent. By combination of STR and SNP markers used, 60 compound HTs could be assigned (cf. Supplementary Table 2 of the Electronic supplementary material). Haplogroup-specific HT diversities were 0.9586±0.0185 for N1c* and 0.9667±0.0195 for I1*. The Y-STR data are available at the YHRD (http://www.yhrd.org) [33].

Altogether, the newly developed typing methods applied to the Turku sample yielded results, which are in accordance with previous observations on Finnish Y-chromosomal lineages [25, 28, 29, 32, 34]. Decreasing the volume of a SNP typing assay in the described manner leads to high-quality results comparable to those observed with conventional typing assays.

Conclusion

The potential to predict the geographical origin of male lineages is a main impetus for analysis of Y-chromosomal polymorphisms in the forensic context [17, 35]. For this study, we designed a RV and a LV assay for Y-SNP typing by minisequencing. Both assays were shown to be highly sensitive and robust as well as being reagent- and time-saving, and there were no inconsistent results between the

two techniques. Both techniques are an interesting option for forensic purposes but they are also of particular interest in population genetic studies due to the high number of samples to investigate. Initial population genetic data obtained with these methods led to similar haplogroup distributions as observed with other typing methods.

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