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RESEARCH NOTE

Multiplex PCR for the assignment of some major branches of the Y chromosome tree

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Introduction

In this paper we describe a multiplex PCR useful to assign some major Y chromosome tree branches (DE, G2, I, J and J2) (for the nomenclature of the Y chromosome tree branches, see YCC 2002). These are prevalent in all Southern European populations, accounting between 51% and 77% of the total male population (Di Giacomo *et al*. 2003 and personal data). The identification of the allelic state at every locus in the study is done either directly (presence/absence of the specific PCR fragment), or can be simply obtained with further sequential steps of dot-blot hybridization with specific probes.

In the last years a large number of single nucleotide polymorphic (SNP) binary markers have been identified in the non-recombining portion of the human Y chromosome (NRY) (YCC 2002). SNPs are ideal genetic markers for medical and population genetic studies, since they are widespread in the human genome and are easily detected by PCR based methods. In general, the SNPs exhibit only two, evolutionarily stable, alleles. This advantage is often counterbalanced by the necessity of typing large numbers of them in many individuals. Genotyping for these markers is of great importance in forensics and in association studies. Moreover, since the NRY lacks genetic recombination during meioses, it is a powerful tool in phylogenetic studies. In order to increase the productivity of such studies, the first choice will be to study simultaneously more SNPs in a single multiplex PCR reaction. Two papers concerning the use of multiplex PCR in the study of the human Y chromosome SNP variation have been published recently. One of them uses a mini-sequencing/capillary electrophoresis method for Y chromosome typing in forensic studies (Sanchez *et al*. 2003); the other one uses a primer extension/mass spectroscopy method for high throughput SNP genotyping of the Y chromosome (Paracchini *et al*. 2002). The method presented here provides a reliable effort-effective, time-effective and cost-effective method for the early stages of human Y chromosome genotyping, being particularly useful when little information or no information at all is available about the genetic structure of the population under study.

Materials and methods

DNA preparation

DNA was isolated from peripheral blood using standard procedures.

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Selection of PCR amplification primers

The Y chromosome markers selected (table 1) to be included in the multiplex reaction were YAP, P15, M170, 12f2, M172 and P27, defining the YCC lineages DE, G2, I, J, J2 and P, respectively. The primers used yielded PCR fragments in the length range 88–455 nucleotides. Due to the repetitive nature of the locus P27 defining the P lineage, the assignment of this lineage using this multiplex is not reliable. However, the multiplex PCR reaction performed much better in the presence of the primers for the P27 locus, so they were included in the multiplex. In a later stage the primers for the P27 locus used in the multiplex reaction were first irradiated at 302 nm, for 5 min, on a standard UV transilluminator. This treatment lead to no amplification of the P27 locus, still assuring the stability of the PCR reaction. For all the primer pairs, their melting temperature and possible interactions were calculated using the publicly available software Primer-Master 1.0 (freely available at ftp://ftp.ebi.ac.uk/pub/software/dos/primer-master).

PCR conditions

The final set-up of the multiplex PCR amplification included 40 ng DNA in a 15 µl reaction volume, containing 1X concentrated PCR buffer (Promega, Madison, WI, USA), 2.5 mM MgCl2 (Promega, Madison, WI, USA), 0.5 mg/ml BSA (Promega, Madison, WI, USA), 200 µM of each dNTP (Promega, Madison, WI, USA), 0.06–0.2 µM of each primer, and 0.75 units of Taq DNA Polymerase (Promega, Madison, WI, USA). All DNA amplifications were performed using an Applied Biosystems GeneAmp 9700 PCR System, with the following cycling conditions: 94°C for 3 min (initial denaturation), followed by 33 cycles for 30 s at 94°C, 30 s at 52°C, and 30 s at 72°C, followed by a final extension for 5 min at 72°C.

Electrophoresis conditions

The PCR products were analysed on 2% agarose gels. TBE(1X) (89 mmol/l Tris base, 89 mmol/l boric acid, 2 mmol/l EDTA, pH8.3) was used as electrophoresis buffer. Gels were stained with 0.5 µg/ml ethidium bromide. The 100 bp ladder from Promega (Madison, WI, USA) was used to assign the size of the PCR fragments. In all cases, the Bio Rad Gel Doc 2000 system was used for the documentation of the gels.

The dot-blot hybridization

After the amplification step, the loci M170, M172 and P15 were further analysed by dot-blot hybridization, with probes designed to specifically detect the allele state at these loci, as elsewhere described (Malaspina *et al*. 2001, Di Giacomo *et al*. 2003). After the detection of one locus was completed, the labelled probes were removed by washing and the detection procedures for another locus were applied.

Multiplex efficiency studies

DNA samples from 84 unrelated males from Romania, Turkey and Greece were first assigned to the six Y chromosome lineages by simplex PCR, followed (where necessary) by dot-blot hybridization. The same samples were then typed with the multiplex PCR and the results of the two assays were compared.

Table 1. Y chromosome markers and primers sequences used in the multiplex PCR reaction.

Locus	PCR Primers (5′ → 3′)	µM	Amplicon size (bp)	No. of matches in the human genome for every primer pair[1]
YAP	Forward: 5′-CAGGGGAAGATAAAGAAATA-3′ Reverse: 5′-ACTGCTAAAAGGGGATGGAT-3′	0.20	455 (YAP +) 150 (YAP –)	107
M170	Forward: 5′-TGCTTCACACAAATGCGTTT-3′ Reverse: 5′-CCAATTACTTTCAACATTTAAGACC-3′	0.10	405	10
M172	Forward: 5′-TTGAAGTTACTTTTATAATCTAATGCTT-3′ Reverse: 5′-ATAATTTATTACTTTACAGTCACAGTGG-3′	0.12	345	9
P27	Forward: 5′-GAACTTGTCGGGAGGCAAT-3′ Reverse: 5′-TGATACACTTCCTCCTTTAGT-3′	0.08	288	16
P15	Forward: 5′-AGAGAGTTTTCTAACAGGGCG-3′ Reverse: 5′-TGGGAATCACTTTTGCAACT-3′	0.06	191	16
12f2	Forward: 5′-GGATCCCTTCCTTACACCTTATAC-3′ Reverse: 5′-CTGACTGATCAAAATGCTTACAGATC-3′	0.14	88	393

[1]The number of matches in the human genome, for every primer pair, was obtained using the software package VPCR2.0 provided as a Web interface (http://grup.cribi.unipd.it/cgi-bin/mateo/vpcr2.cgi).

Results and discussion

Electrophoresis and detection of PCR products

The assignment of the DE lineage was done directly by examining the gels for the presence of the appropriate PCR product (the samples carrying the YAP insertion, thus belonging to lineages DE, yielded a 455 bp fragment, while the samples without the YAP insertion yielded a 150 bp fragment). The assignment of the J lineage was also done directly by examining the gels for the presence or absence of the appropriate PCR product. The 12f2(+) samples (samples not belonging to the J lineage) yielded an 88 bp PCR product, while the 12f2(–) samples (samples belonging to the J lineage) yielded no product at all. In this case, the presence of the other amplified PCR products in that sample served as an internal amplification control. The other four markers present in the multiplex reaction (M170, M172, P27, and P15) yielded PCR products of 405 bp, 345 bp, 288 bp, and 191 bp in length, respectively (see figure 1).

Primer concentration in the PCR reaction

It was necessary to adjust the primer concentration to obtain balanced PCR products mix. The final concentration of primers ranged from 0.06 to 0.2 µM.

PCR adjuvants

We found that the best results of amplification of all DNA targets were obtained in the presence of BSA (Bovine Serum Albumin) in a concentration of 0.5 mg/ml.

The dot-blot hybridization

The assignment of the M170, M172 and P15 loci was done by dot-blot hybridization after the spotting of the final PCR products mix on nylon membranes. The detection procedures for every locus did not affect the detection efficiency of the other loci co-amplified in the multiplex.

Efficiency of the Y chromosome typing with the multiplex PCR

SNP typing was performed in 84 unrelated males from Romania, Turkey and Greece, by simplex PCR, followed by dot-blot hybridization (where necessary), then by the multiplex assay described here. In all cases, consistent results were obtained.

Discussion

We designed a multiplex PCR reaction for the easy assignment of five lineages (DE, G2, I, J and J2) of the Y chromosome tree. Markers published by Underhill *et al*. (2001) (M170, M172), Rosser *et al*. (2000) (12f2) and Hammer and Horai (1995) (YAP) and Hammer *et al*. (2000) (P15, P27) were co-amplified in the same PCR reaction, in order to develop a multiplex PCR typing method specifically designed for Southern European populations, where these lineages account for 51% to 77% of the total male population (Di Giacomo *et al*. 2003 and personal data). Two of these lineages (DE and J) are assigned directly, the other three (G2, I and J2) after a further step of dot-blot hybridization. Furthermore, after the M172 hybridization results, samples ancestral for M172 and derived for 12f2 are assigned as J*(xJ2).

Successful PCR multiplexing depends on a number of factors, like the nature and concentration of the PCR buffer, the $MgCl_2$ and dNTP concentration and balance, the annealing temperature, the extension time, the nature and concentration of various possible adjuvants, etc (Henegariu *et al*. 1997). Here we present some consideration regarding the multiplex PCR reaction we set up.

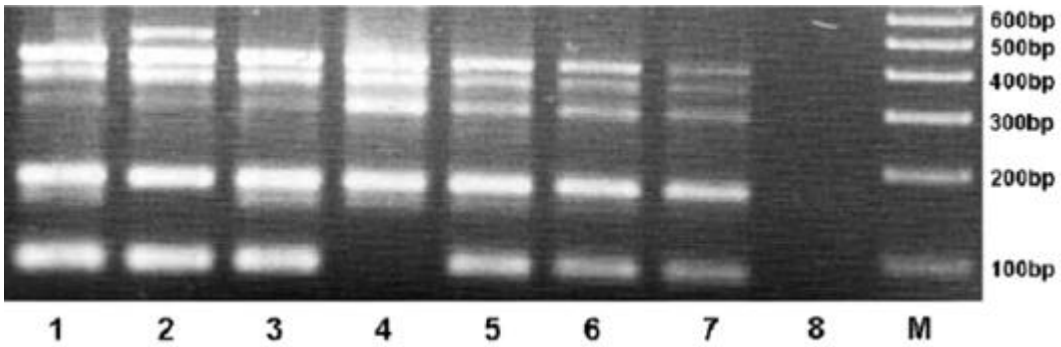

Figure 1. Agarose gel (2%) of multiplex PCR reaction with the markers YAP, P15, M170, M172, 12f2 and P27. The fragments yielded for every locus: 455 bp (YAP +), 405 bp (M170), 345 bp (M172), 288 bp (P27), 191 bp (P15), 150 bp (YAP –), 88 bp (12f2 +). Lane 1, 3, 5, 7: YAP(–), 12f2(+) individuals; the PCR fragments (from top to bottom): M170, M172, P27, P15, YAP(–), 12f2. Lane 2: YAP +, 12f2(+) individual; the PCR fragments (from top to bottom): YAP(+), M170, M172, P27, P15, 12f2. Lane 4: YAP(–), 12f2(–) individual; the PCR fragments (from top to bottom): M170, M172, P27, P15, YAP(–). Lane 8: female sample showing no amplification products. M: 100 bp Ladder (Promega, Madison, WI, USA).

At DNA template quantities between 40 and 300 ng/15 µl reaction volume, the amplification reaction showed no significant differences; however, below 40 ng the amount of some of the PCR products (12f2, M170 and M172) decreased. The PCR buffer contained KCl, Tris, and Triton X-100. The optimum $MgCl_2$ concentration we found was 2.5 mM; higher concentration of MgCl2 yielded an increase of the concentration of products of unexpected molecular weight (data not shown). The use of BSA led to an increased specificity of the PCR reaction. The presence/absence of the primers for the P27 locus in the multiplex reaction proved to be important for the stability of the reaction. Even in the presence of P27 primers, irradiated at 302 nm on standard transilluminator, the PCR reaction performed well, with no longer amplification of the P27 marker, while in the absence of these primers, the reaction yielded large number of unspecific products. The concentration of the PCR primers in the final multiplex reaction ranged from 0.06 to 0.2 µM. The different necessary concentration for every primer pair is probably connected, among other possible factors, with the number of targets in the genomic DNA. To test this hypothesis we used the software packages VPCR2.0 provided as a web interface (http://grup.cribi.unipd.it/cgi-bin/mateo/vpcr2.cgi). Testing how many matches there are in the human genome for every pair of primers used in the multiplex, we obtained the values listed in table 1. These results could suggest the need for a higher concentration required by the YAP and 12f2 primers; however, there is not a clear correlation between the number of matches and the required concentration determined experimentally for every primer pair. This situation supports the idea that the concentration of the primers in a multiplex PCR reaction is influenced by a number of factors (number of targets, quality of template) including some subtle unknown interactions.

We used the multiplex PCR method presented to type Southern European populations (from Italy, Greece, Crete, Romania, Moldavia Republic, Bulgaria, Albania, Turkey) and the allele frequencies obtained for every locus were in accordance with data in literature, where available (Malaspina *et al*. 1998; Rosser *et al*. 2000; Semino *et al*. 2000; Stefan *et al*. 2001; Di Giacomo *et al*. 2003).

This multiplex PCR approach provides a time-effective and effort-effective Y chromosome typing strategy, being easier and faster than the classical step-by-step analysis of every locus. Moreover, because of the simple dot-blot hybridization procedures used to further analyse the samples, the method is cheaper than other multiplex PCR based methods existent in the literature. It is to be observed that alternative methods of allele detection can be applied to the products obtained with this method. For example allele-specific nested PCR reaction for use in automated sequencers are promising for hastening the detection step. The method is particularly useful for typing Southern-European populations, where the five analysed lineages account for 51% to 77% of the total male population.

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