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TECHNICAL ADVANCES

New generation sequencers as a tool for genotyping of highly polymorphic multilocus MHC system

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Abstract

Accurate genotyping of complex systems, such as the major histocompatibility complex (MHC) often requires simultaneous analysis of multiple co-amplifying loci. Here we explore the utility of the massively parallel 454 sequencing method as a universal tool for genotyping complex MHC systems in nonmodel vertebrates. The power of this approach stems from the use of tagged polymerase chain reaction (PCR) primers to identify individual amplicons which can be simultaneously sequenced to the arbitrarily chosen coverage. However, the error-prone sequencing technology poses considerable challenges as it may be difficult to discriminate between sequencing errors and true rare alleles; due to complex nature of artefacts and errors, efficient quality control is required. Nevertheless, our study demonstrates the parallel 454 sequencing can be an efficient genotyping platform for MHC and provides an alternative to classical genotyping methods. We introduced procedures to identify the threshold that can be used to reduce number of genotyping errors by eliminating most of artefactual alleles (AA) representing PCR or sequencing errors. Our procedures are based on two expectations: first, that AA should be relatively rare, both overall and on per-individual basis, and second, that most AA result from errors introduced to sequences of true alleles. In our data set, alleles with an average per-individual frequency below 3% most likely represented artefacts. This threshold will vary in other applications according to the complexity of the genotyped system. We strongly suggest direct assessment of genotyping error in every experiment by running a fraction of duplicates: individuals amplified in independent PCRs.

Keywords: genotyping, high throughput sequencing, MHC, pyrosequencing, sequencing errors

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Introduction

Genotyping of complex multilocus systems such as the major histocompatibility complex (MHC) is a demanding task. While in humans and a few other model organisms standardized approaches exist (Bunce *et al*. 1995; Susskind 2007), there are many species for which accurate genotyping poses a considerable challenge. Studies on MHC variation in nonmodel vertebrates are important for understanding their adaptive significance in the wild and for conservation purposes (Bernatchez & Landry 2003; Sommer 2005).

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Genomic structure of the major histocompatibility complex is highly variable across vertebrates (Kelley *et al*. 2005). Frequently, multiple loci resulting from recent duplications are present. Allelic lineages, and even alleles, may be shared among loci, possibly as a result of interlocus recombination and gene conversion (Ellis *et al*. 1999; Van Oosterhout *et al*. 2006). In such cases, locus-specific amplification and genotyping may not be possible and simultaneous genotyping of multiple loci becomes necessary. While several techniques based on physical separation of alleles are available, such as single-stranded conformational polymorphism (SSCP), denaturing gel gradient electrophoresis (DGGE), or reference strand mediated conformational polymorphism (RSCA), these are increasingly difficult to use and unreliable when number of alleles and

loci is high. Also, when available primers amplify certain alleles with lower efficiency, such alleles will likely be missed by these genotyping assays, as the signal produced by such alleles will be undistinguishable from the background noise. Another difficulty with indirect methods is technical complexity of the genotyping process, usually involving several preparatory steps such as identification, isolation and sequencing of alleles, e.g. by cloning or sequencing of excised SSCP or DGGE bands. Indirect genotypic methods usually have limited resolution, and thus, may not distinguish some variants, compromising quality of genotyping. Direct sequencing of PCR products with subsequent computational inference of alleles from diploid sequences which has been proved efficient in the case of single-locus variation (Bos *et al*. 2007) is not suitable when more than two alleles co-amplify.

Prompted by complexity revealed by the studies of the major histocompatibility complex (MHC II) DRB variation in the bank vole (Axtner & Sommer 2007; Babik & Radwan 2007), a rodent species extensively studied by ecologists and evolutionary biologists (e.g. Radwan *et al*. 2004; Sadowska *et al*. 2005; Mills *et al*. 2007), we decided to explore utility of the massively parallel 454 sequencing method as a universal tool for genotyping complex MHC systems in nonmodel vertebrates. The method (Margulies *et al*. 2005) is capable of providing *c*. 1 million sequences in a single instrument run in its most recent implementation. The sequences are 400–500 bp each, which is more than sufficient for genotyping of the most variable second exon of MHC II genes. Sequences are effectively derived from a single DNA molecule each. Basically, this method is equivalent to sequencing of clonally amplified products derived from a single molecule in a cell-free system, thus avoiding artefacts related to cloning in bacteria (Longeri *et al*. 2002). By using individually coded, 'tagged' primers for polymerase chain reaction (PCR) amplification of the desired products, it is possible to sequence in a single 454 experiment PCR products derived from tens or hundreds of individuals (Binladen *et al*. 2007; Meyer *et al*. 2008). Because sequencing is performed at a high coverage, the level of which can be easily adjusted as required, it is possible to obtain accurate representation of all variants present in a given amplicon, even those which are present in a low number of copies (Thomas *et al*. 2006).

However, before the potential of 454 technology for genotyping of complex MHC systems can be fully realized a crucial issue of accuracy needs to be addressed. The rate of sequencing errors of the system is higher than that of the classical Sanger sequencing and highly dependent on the sequence context (Moore *et al*. 2006; Brockman *et al*. 2008). Particularly prone to sequencing errors are homopolymer runs and, interestingly, also regions adjacent to them (Moore *et al*. 2006; Brockman *et al*. 2008). Most of errors in these regions constitute over- or undercalls resulting in a considerable frequency of indels in sequencing reads. There has been a considerable recent interest in quantifying sequencing errors, understanding of their sources and devising adequate quality control procedures in 454 sequencing (Moore *et al*. 2006; Huse *et al*. 2007; Brockman *et al*. 2008). This problem is particularly severe in the case of highly polymorphic, multilocus systems such as MHC, as it may be difficult to discriminate between sequencing errors and true rare alleles. Here, we attempt such discrimination by examining frequency distribution of sequence variants and their similarity to the most common variants.

Materials and methods

Laboratory procedures and initial quality control

We designed degenerate primers MgDRBL (5′GACAGAKACWTCTACAAYCRG3′) and MgDRBR (5′-TAGTTGTRTCTGCAGWAYGYGTCC-3′) located in conserved regions of the second MHC II exon of the bank voles. These conserved regions were chosen on the basis of extensive sequence data available (Axtner & Sommer 2007; Babik & Radwan 2007). The forward primer started with CC followed by a 4-bp tag identifying an individual, and the sequence of the primer MgDRBL. CC dinucleotide at the 5′-end of primers was used to eliminate the effect of the 5′-terminal nucleotide on the tag efficiency (Binladen *et al*. 2007; Valentini *et al*. 2009). To genotype 96 bank voles, we used 96 out of 108 possible tags which did not contain two identical bases in adjacent positions. PCR was performed in 15 μL reactions using HotStar MasterMix (QIAGEN), 1 μM of each primer, and 20–50 ng of genomic DNA. The cycling scheme was: 94 °C for 15 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 3 min. PCR products were purified using MinElute PCR Purification Kit (QIAGEN), their concentrations measured with Nanodrop-1000 spectrophotometer and adjusted accordingly. Equimolar amounts of individual amplicons were then pooled and the pool was sequenced using a part of a single 454 FLX instrument sequencing run (13,953 sequences).

After the initial quality assessment using standard settings of the 454 software, the following procedure was used to increase stringency of the quality control. Only sequences of at least 120 bp (excluding primers) showing perfect match to the forward primer, containing a complete tag, first 12 bases of the reverse primer and not showing any N calls in the target region were extracted from the multifasta file produced by 454 using a custom program available from the website (www.eko.uj.edu.pl/radwan). Sequences with any N-calls were removed because they have been shown to be a signature of overall poor read quality (Huse *et al*. 2007). Unique sequences were identified and sorted according to the tag they displayed.

Identification of sequencing artefacts

Despite strict quality control applied to our data, a fraction of sequences might still contain errors and could be misidentified as rare true alleles. We developed procedures which should allow identification and exclusion of such artefactual alleles (AA). At the core of our reasoning are two expectations: first, AA should be relatively rare, both overall and on per individual basis; second, all AA result from errors introduced to sequences of true alleles (TA). Therefore if we rank alleles according to their relative frequency in the data set, there should be a threshold below which mostly or exclusively AA occur. To identify this threshold, we adopted two approaches, one based on similarity to known alleles and the other on the average per-individual frequency. Both are certainly related, but each explores a different aspect of the data. Thus, similar thresholds suggested by both approaches would demonstrate correct identification of AA.

Similarity to known alleles. The rationale of this approach was as follows. We assume that 454 sequencing errors are of the type of undercalls, overcalls (both causing indels), or substitutions, and PCR errors are mainly substitutions introduced to the sequences of TA, although PCR recombinants may occasionally occur as well (Zylstra *et al*. 1998). Consequently, AA should be similar to TA. As MHC allelic lineages are maintained by balancing selection over long evolutionary periods, and therefore are often highly divergent, TA should be on average less similar to some TA present in a given individual than AA are similar to TA. Hence, when we apply some metrics measuring this (dis)similarity, their average values should be different in AA and TA.

We used three distance metrics which should be correlated with each other but nevertheless reveal different aspects of the data. For each individual, we divided its alleles into two groups: four most common alleles (FMCA) and the remaining alleles (RA). As the presence of at least four MHC II loci has been postulated in the bank vole (Axtner & Sommer 2007), choosing the four most common alleles is the conservative approach ensuring presence of only true alleles in the first group. Then, for each of the RA, we computed a minimum distance to any of the FMCA according to the three metrics:

1 IND metric. Indels are the most frequent 454 sequencing errors (Huse *et al*. 2007) yet they should not be particularly common among true MHC alleles as only minority of them seem to represent pseudogenes which are free to accumulate indels (Axtner & Sommer 2007; Babik & Radwan 2007); if a RA differed from any of the FMCA only by indel(s), such allele was assigned score 0 in a given individual, otherwise 1.

2 SUB metric for an allele in an individual was given a value 0 if this allele differed by at most two substitutions from any of FMCA.

3 DIS was simply the number of substitutions from a given allele to the most similar of the FMCA.

Values of each metric for each allele were averages across all individuals possessing the allele. There should be no correlation between the allele rank (relative frequency) and distance metrics neither within TA nor for AA, but, as MHC alleles typically show high levels of divergence, average distance from FMCA should be higher in TA. Therefore, we predict that there should be a significant negative correlation between the rank and distance at the transition between frequency classes comprising mostly TA and mostly AA. Another qualitative prediction was that there should be a very strong, visually detectable change in the IND metric between TA and AA.

Correlation between allele rank and all metrics was examined by a sliding-window analysis with the window size of 30 and the step of 20.

Per-individual frequency. Our second approach is based on the expectation that AA should be rare on the per-individual basis. Even if a given AA is repeatedly produced via errors introduced to the sequence of a common TA, and therefore, its overall frequency in the data set might be appreciable, it should still be rare on the per-individual basis. We computed the average per-individual frequency of each allele (taking into account only individuals in which the allele was present) and plotted it against the allele rank.

Results and discussion

A total of 11 022 sequences conformed to our initial quality criteria. These represented 1859 unique DNA sequences in the range of 120–129 bp (excluding primers). We used 96 out of 108 possible tags which did not contain two identical bases in adjacent positions. However, theoretically we could expect that any of 256 possible tetranucleotide tags may be present in the data set because of errors during sequencing or primer synthesis. After the initial filtering, 10 sequences with tags not matching any of the tags used in the experiment were found, which is 0.091% of the total number of sequences. As probability of a substitution not matching any of the tags used is approximately the proportion of unused tags in all tags possible (here 160 in 256), the level of misassignment of alleles to individuals is low, *c*. 0.145%, as noticed previously (Parameswaran *et al*. 2007).

To further filter out PCR and sequencing artefacts, we applied another criterion: to be included in subsequent analyses, a sequence had to be present in PCR products

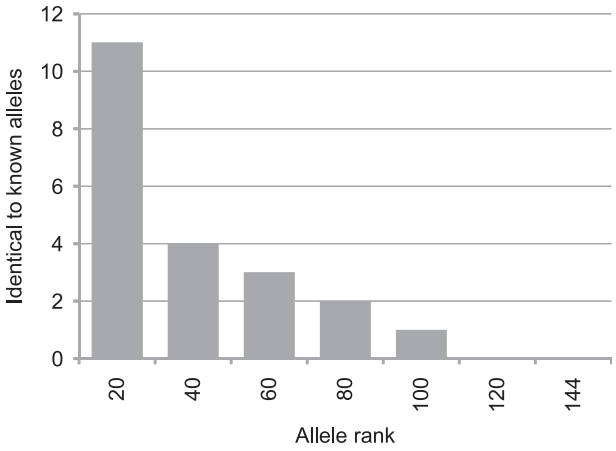

Fig. 1 Number of alleles identified and confirmed in earlier studies (Axtner & Sommer 2007; Babik & Radwan 2007) among 144 unique sequences detected in the present study ranked according to their abundance.

derived from at least two PCRs (identified by tag sequences), represented by two or more sequences in at least one of them, or had to be present in at least three independent PCRs. This criterion is conservative because it automatically excludes alleles present in a single individual only, regardless of the number of copies. It is necessary, however, to exclude artefacts produced during PCR, which is a standard procedure adopted in MHC studies (e.g. Babik *et al*. 2005).

The data set trimmed according to the above criteria comprised 8614 sequences, of which 144 were unique. Despite standardization of PCR product concentration before pooling of individual amplicons, the number of sequences obtained per individual varied over two orders of magnitude. For one of 96 individuals genotyped, no sequences meeting quality criteria were obtained, and only single sequences were available for three individuals, but the next lowest count was 16. The highest observed number was 242 sequences, and the per-individual average (excluding counts 0 and 1) was 93.6 sequences (SD = 44.4). Other studies also report substantial heterogeneity of coverage among amplicons even in the case of careful standardization of concentrations (Brockman *et al*. 2008).

Because the fragment considered here was shorter than the length of alleles reported so far, the 37 alleles described (Babik *et al*. 2005; Axtner & Sommer 2007) were reduced to 32 unique sequences, because some polymorphisms distinguishing originally described alleles were located in fragments not covered by our sequences. Twenty-one alleles in our data set were identical to the already-known bank vole alleles (44.9% of reads). However, when indel positions were excluded from the analysis, an additional 23 alleles (representing 4.1% of all reads) matched known alleles, and only one of them did not match any of the 21 sequences identified before indels were excluded. Thus, artefacts resulting from over- and undercalls appear common in our data set.

Very high number of unique sequences detected and the pattern of their similarity (many sequences differing by 1–2 substitutions or indels) strongly suggested that despite stringent quality control, our data set still contained artefacts, resulting from errors of the kinds unaccounted for by our filtering procedures. A remarkable outcome has been observed when all 144 putative alleles were ranked according to their number of copies in the data set (Fig. 1). Almost all alleles identical to already-known alleles ranked high, all but one case ≤ 66, and thus, virtually no alleles from the second half of the distribution have been confirmed by their identity to already-known alleles (Fig. 1). This in principle could indicate that alleles of high ranks are indeed rare true alleles; however, as rare alleles (present in a single individual only) were already excluded, a more likely reason for such skewed distribution is that alleles of high ranks are in fact mostly PCR and/or sequencing artefacts.

The prediction that there should be a significant negative correlation between the rank and our similarity metrics at some intermediate frequency range was confirmed by our data. For both IND and SUB metrics significant negative correlation is observed only in the allele frequency rank of 61–90 (for IND $R^2 = 0.292$, $n = 30$, $P = 0.002$, for SUB $R^2 = 0.190$, $n = 30$, $P = 0.016$), particularly abrupt shift in IND metric was observed around allele rank 74 (Fig. 2); in the case of DIS metric, correlation in the same window was close to significant ($R^2 = 0.117$, $n = 30$, $P = 0.064$), whereas no indication of any correlation was found in other windows. All alleles ranked 74 and higher were present in the whole data set in 15 or fewer copies although some of them were present in a considerable number of independent PCRs – up to 11 in the case of the allele 74. All metrics were much lower for alleles ranked 74 and higher (Mann–Whitney *U* tests, all *P*s $< 10^{-4}$).

Inspection of the plot of per-individual frequency against allele rank (Fig. 3) clearly demonstrates that only three alleles above rank 73 cross the threshold of 3% average per-individual frequency; on the contrary, only five alleles below rank 74 fall below that threshold.

The agreement between the results of our two approaches used to identify AA threshold is remarkable. Both identify the threshold separating predominantly TA from AA between alleles ranked 73 and 74. This suggests that sequencing errors producing repeatedly and probably nonrandomly AAs from TAs are a major source of artefacts in 454 MHC II DRB genotyping in the bank vole. In the following assessment of genotype diversity, we decided to treat as TA only alleles ranked 1–73. The 3% per-individual frequency threshold for AA identification (Fig. 3) seems reasonable, given the complexity of the MHC system in

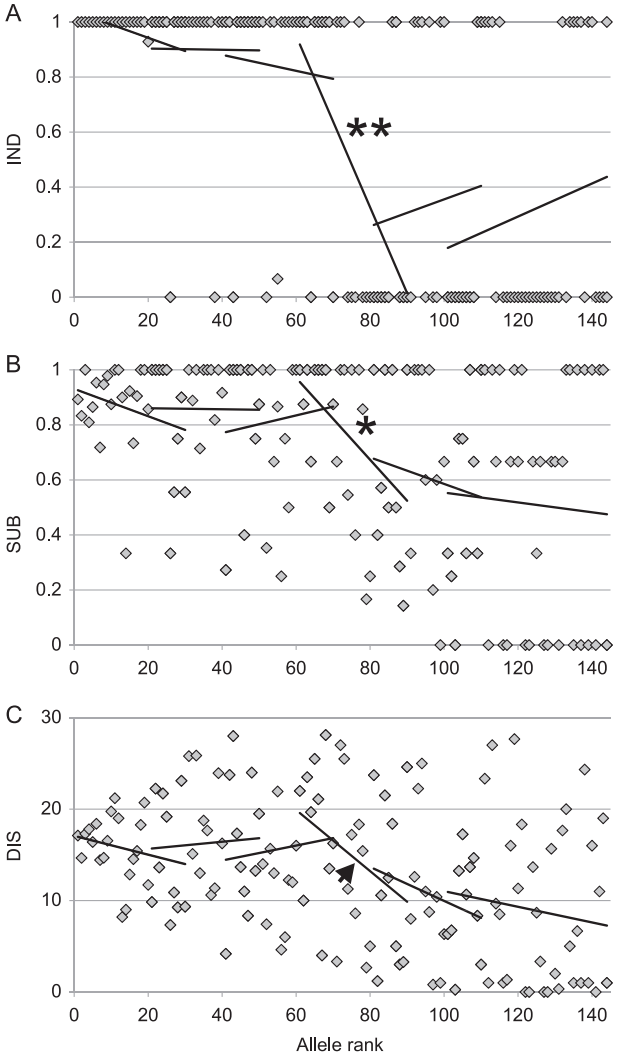

Fig. 2 Results of the sliding window analysis of a correlation between the allele rank (proxy for abundance) and three metrics reflecting distance of the allele from individual's four most common alleles: (A) IND, (B) SUB, (C) DIS. The scores of each allele on the *y* axes are averages across individuals. The only significant correlations between metrics and allele rank were observed in the window 61–90 for IND ($P = 0.002$) and SUB ($P = 0.016$), indicated with asterisks, the correlation for DIS was approaching significance ($P = 0.064$), indicated with an arrow.

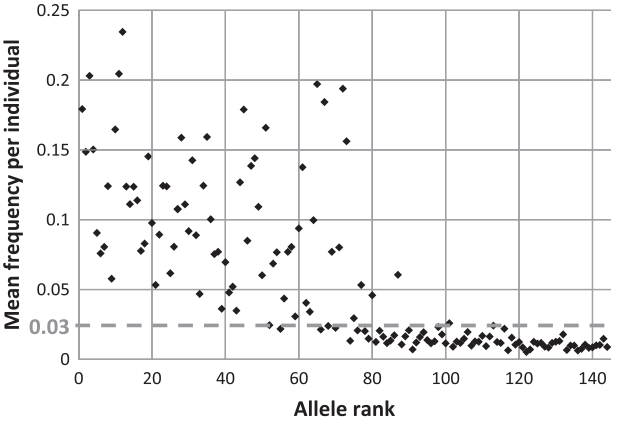

Fig. 3 Relationship between the mean per-individual allele frequency (taking into account only individuals in which the allele was present) and the allele rank (relative frequency in the data set) for 144 DRB alleles conforming to initial quality criteria. The threshold of 3%, separating the true alleles from the artefactual alleles is indicated.

the bank vole. In other studies using pyrosequencing for genotyping multilocus systems, the threshold level should vary according to their complexity.

The 73 alleles retained after the final filtering were represented by 8224 sequences. A threshold of 50 sequences per individual was applied for genotyping; 79 individuals met this criterion. The number of alleles per individual ranged from 4 to 18 with the arithmetic mean (AM) of 10 and the mean weighted by the number of sequences (WM) 10.3. If only alleles detected in at least two sequences per individual were included, the numbers were 3 to 18 with AM of 8.8 and WM 8.5. Although rather poorly resolved due to short length of available sequences, a phylogenetic tree used for visualization purposes (not shown) indicated detection of several groups of MHC II related sequences not reported earlier in the bank vole. Because we used for amplification degenerate primers located in conservative portions of the 2nd DRB exon, it could be expected that these divergent sequences represent MHC II genes other than DRB. In all cases however, BLAST searches revealed DRB sequences as closest matches. On the other hand, of 32 known alleles, 11 were not confirmed in our data set.

The cost of the current approach is highly dependent on the experimental design and includes cost of tagged primers which may be substantial. However, in the case of the bank vole, in which the 400× coverage of an amplicon should be more than sufficient, genotyping of 1000 individuals should be possible in a single 454 FLX run, which gives the cost of approximately 10 € per individual. This cost is likely to drop significantly in the near future with the increase of per run output promised by the Titanium technology.

Presence of 18 unique alleles implies the existence of at least nine polymorphic MHC loci. This number is much higher than reported in previous studies on the bank vole (Axtner & Sommer 2007; Babik & Radwan 2007), clearly emphasizing the need for accurate genotyping methods and for careful design of primers capable of capturing as much variation as possible. High variation in the number of alleles detected per individual may indicate: (i) non-amplification of some alleles—unlikely because our primers were designed on the basis of extensive information on

sequence polymorphism to be as universal as possible; (ii) variation among individuals in the number of loci (Sato *et al*. 1998; Bowen *et al*. 2004), (iii) allele-sharing among loci (Miller & Lambert 2004; Van Oosterhout *et al*. 2006), (iv) random effects resulting from insufficient coverage. As expected, we observed a positive correlation between the number of available sequences and the number of alleles detected per individual (data not shown). This is easily understood because considering the mean number of alleles detected per individual of 10, the coverage on the per-allele basis was usually of the order of only 5–10×.

Our study reveals complex nature of 454 sequencing errors with respect to genotyping of complex multilocus systems amplified using the PCR method. Although analyses based on correlations and theoretical expectations provide valuable insights, it is obvious that the critical test of genotyping quality would involve empirical assessment of genotyping error via running duplicates: a fraction of samples should be amplified in two independent PCRs with two differently tagged primers and sequenced. We recommend that such an estimate of experimental error should be performed in every experiment or set of experiments (e.g. Huse *et al*. 2007) perhaps with the use of more efficient quality scores than routinely used (Brockman *et al*. 2008). With the appropriate empirical quality control, genotyping of complex multigene systems should be easily accomplished in any nonmodel organism for which enough sequence information is available to design appropriate primers.

Conclusions

In conclusion, the present study demonstrated that 454 parallel pyrosequencing can be an efficient genotyping platform for complex multilocus systems such as MHC in nonmodel vertebrates and provides an alternative to classical genotyping methods. However, due to the complex nature of artefacts and errors introduced by this technique, efficient quality control is required. We introduced procedures which allowed us to identify the threshold that can be used to reduce number of genotyping errors by eliminating most of artefactual alleles (AA) representing PCR or sequencing errors. Our procedures were based on two expectations: first, that AA should be relatively rare, both overall and on a per-individual basis, and second, that most AA result from errors introduced to sequences of true alleles. In our data set, alleles with an average per-individual frequency below 3% most likely represented artefacts. This threshold will vary in other applications according to the complexity of the genotyped system, i.e. the number of alleles expected per individual. We strongly suggest direct assessment of genotyping error in every experiment by running a fraction of duplicates: individuals amplified in independent PCRs.

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