DMD Fast Forward. Published on January 13, 2006 as DOI: 10.1124/dmd.105.008292
DMD Fast Forward. Published on January 31, 2006 as doi:10.1124/dmd.105.008292
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DMD #8292

*CYP2D6*36* gene arrangements within the *CYP2D6* locus: Association of *CYP2D6*36* with poor metabolizer status

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Running title: *CYP2D6*36* arrangements within the *CYP2D6* gene locus

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Number of text pages 27

Number of tables: 3

Number of figures: 3

Number of references: 21

Number of words in the *Abstract*: 247

Number of words in the *Introduction*: 534

Number of words in the *Discussion*: 964

List of non-standard abbreviations: CYP2D6, cytochrome P450 2D6; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer;

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Abstract

Unexplained cases of CYP2D6 genotype/phenotype discordance continue to be discovered. In previous studies several African Americans with a poor metabolizer phenotype carried the reduced function *CYP2D6*10* allele in combination with a nonfunctional allele. We pursued the possibility that these alleles harbor either a known sequence variation (i.e. *CYP2D6*36* carrying a gene conversion in exon 9 along the *CYP2D6*10*-defining 100C>T SNP*)* or novel sequences variation(s). Discordant cases were evaluated by long-range PCR to test for gene rearrangement events and a 6.6 kb PCR product encompassing the *CYP2D6* gene was cloned and entirely sequenced. Subsequently, allele frequencies were determined in different study populations comprising Caucasians, African Americans and Asians. Analyses covering the *CYP2D7-2D6* gene region established that *CYP2D6*36* did not only exist as a gene duplication (*CYP2D6*36x2*) or in tandem with **10* (*CYP2D6*36+*10*), as previously reported, but also by itself. This 'single' *CYP2D6*36* allele was found in nine African Americans and one Asian, but was absent in the Caucasians tested. Ultimately, the presence of *CYP2D6*36* resolved genotype/phenotype discordance in three cases. We also discovered an exon 9 conversion-positive *CYP2D6*4* gene in a duplication arrangement (*CYP2D6*4Nx2*) and a *CYP2D6*4* allele lacking 100C>T (*CYP2D6*4M*) in two Caucasian subjects. The discovery of an allele that carries only one *CYP2D6*36* gene copy provides unequivocal evidence that both, *CYP2D6*36* and **36x2* are associated with a poor metabolizer phenotype. Given a combined frequency of between 0.5-3% in African Americans and Asians genotyping for *CYP2D6*36* should improve the accuracy of genotype-based phenotype prediction in these populations.

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Cytochrome P450 2D6 (CYP2D6) is a major drug metabolizing enzyme involved in the biotransformation of many clinically important medications, including antidepressant and antipsychotic drugs (Kirchheiner et al., 2004). CYP2D6 activity ranges considerably within a population and includes individuals with ultrarapid (UM), extensive (EM), intermediate (IM) and poor (PM) metabolizer status. Genetic variation within the *CYP2D6* gene locus is the major contributing factor determining a subject's activity or capability to metabolize certain drugs (Zanger et al., 2004). The *CYP2D* gene locus on chromosome 22 contains three contiguous genes, *CYP2D8*, *CYP2D7* and *CYP2D6* (Kimura et al., 1989) with only the latter encoding a functional protein (Gaedigk et al., 2005c). The *CYP2D6* gene is not only highly polymorphic with 58 allelic variants and numerous subvariants reported to date (http://www.imm.ki.se/CYPalleles; accessed Jan 5, 2006), but rearrangements within the gene locus have created alleles harboring two or multiple *CYP2D6* genes, deleted the entire gene or led to the creation of fused *CYP2D7/2D6* genes. Despite vast efforts characterizing *CYP2D6* allelic variants in many populations of different ethnicity, subjects exhibiting a phenotype that is discordant with their determined genotype persist. Most of these cases are poor metabolizers who carry at least one functional or reduced-function allele predicting an extensive or intermediate metabolizer phenotype. Follow-up studies of such cases have revealed novel alleles such as *CYP2D6*40* and **42* in African Americans (Gaedigk et al., 2002; Gaedigk et al., 2003a) and *CYP2D6*21* and **44* in Japanese (Yamazaki et al., 2003).

*CYP2D6*36*, originally termed Ch2 or **10C* (http://www.imm.ki.se/CYPallele) has been described in a tandem arrangement with *CYP2D6*10B (CYP2D6*36* located upstream of *CYP2D6*10B*, Fig. 1) (Johansson et al., 1994). Regarding CYP2D6.36 function, Johansson et al. (Johansson et al., 1994) speculated that the 100C>T SNP ($P_{34}S$) present in *CYP2D6*10* and **36*

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is the major cause for protein destabilization and that the six amino acids difference in exon 9 (i.e. the *CYP2D7* exon 9 conversion) do not have a significant impact on CYP2D6.36 activity. Fukuda et al. (Fukuda et al., 2000) later demonstrated that decreased clearance by CYP2D6.10 and CYP2D6.36 is not only caused by low protein expression, but also increased Km values. However, since the *CYP2D6*36* gene did not appear to occur by itself and activity was conferred by the downstream *CYP2D6*10* gene on this allele, no further functional studied were pursued, and thus it remained unclear whether *CYP2D6*36* was an IM or PM-associated allele. However, a discordant Japanese case was recently resolved by the discovery of a *CYP2D6*36x2* gene duplication demonstrating that *CYP2D6*36* is a loss-of-function allele as assessed by phenotyping with the probe drug debrisoquine (Chida et al., 2002).

Current genotyping protocols, including our own, define *CYP2D6*10* and **36* by the presence and absence of two SNPs, 100C>T and 1846G>A, respectively. Consequently, the known **10A/B, *10x2, *36x2* and **36+*10* (tandem) alleles (Fig. 1) are not discriminated and are collectively genotyped as *"CYP2D6*10"*. Interestingly, three of our discordant cases had a "*CYP2D6*10"* in combination with a non-functional *CYP2D6*4* allele and we hypothesized that **"***CYP2D6*10"* was in fact a masked *CYP2D6*36x2* allele as described by Chida et al. (Chida et al., 2002).

To test this hypothesis, we conducted a detailed characterization of the *CYP2D6/2D7* gene locus in phenotypic poor metabolizers with an assigned *CYP2D6*10* allele.

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Methods

Subjects. Study protocols, blood collection and use of tissues were approved by the Missouri-Kansas City Adult and Pediatric Health Sciences Review Boards and the Morehouse School of Medicine Review Board (Atlanta, GA), respectively. All study participants gave written informed consent.

Subjects were phenotyped with dextromethorphan and genotyped previously. These studies comprised adult Caucasian Americans, n=214 (Gaedigk et al., 1999) and African Americans, n=281 (Gaedigk et al., 2002); African Americans with sickle cell disease (children > 1 years of age), n=126 (Alander et al., 2002); and an ethnically diverse pediatric population (subjects enrolled at 2 weeks of age), n=155 (Blake et al., manuscript in preparation).

An additional 81 genomic DNA samples were derived from an ethnically diverse tissue collection (ethnicity data were retrieved from data sheets accompanying the tissues) that was obtained from NICHD-supported tissue retrieval programs - the University of Maryland Brain and Tissue Bank for Developmental Disorders, (Baltimore, MD), and the Central Laboratory for Human Embryology at the University of Washington (Seattle, WA). DNA samples of Asian Americans, n=38, were collected for a previous study (Gaedigk et al., 1999) or from discarded, anticoagulated blood obtained for routine clinical management of hospitalized patients (self-reported ethnicity on hospital admission from).

DNA isolation and CYP2D6 genotyping. DNA was isolated from whole blood or liver tissue using DNA blood and DNeasy tissue kits (Qiagen, Valencia, CA), respectively. A subset of DNAs were isolated from cheek scrapings (buccal brushes) using Gentra reagents as recommended (Gentra Systems, Minneaplois, MN). *CYP2D6* genotyping was performed as

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previously described (Gaedigk et al., 1999; Gaedigk et al., 2002; Gaedigk et al., 2003a; Gaedigk et al., 2003b; Gaedigk et al., 2005a; Gaedigk et al., 2005b) and included **2* through **12, *14, *15, *17, *28, *29, *35, *40, *41, *42, *45, *46* and **1x2, *2x2,* and **4x2* gene duplications. *CYP2D6* allele nomenclature throughout this report uses the recently revised allele definitions as established by the *CYP2D6* nomenclature committee (http://www.imm.ki.se/CYPalleles).

Cloning and sequencing of CYP2D6 alleles. A 6.6 kb *CYP2D6*-specific fragment that encompassed the entire gene (Fig. 1, fragment C) was amplified and cloned with the pCR-XL-TOPO cloning kit (Invitrogen, Carlsbad, CA). This fragment is only derived from the most downstream gene within the locus and was also used as genotyping template. Sequence analysis of clones was performed with DYEnamic ET dye terminator chemistry and a MegaBACE 500 capillary sequencer (Amersham Biosciences, Piscataway, NJ). AY545216 served as *CYP2D6*1* reference sequence (Gaedigk et al., 2005a).

PCR reactions and conditions. Primer sequences and additional details pertaining to all PCR reactions performed for the study are presented in Table 1.

Long-range PCR: Reactions for PCR fragments A to D were carried out with JumpStart REDAccuTaq LA DNA Polymerase (Sigma, St. Louis, MO) in the presence of 5% DMSO. Reaction volumes were 8 µl and composition as recommended. Extension times were 11, 12, 7 and 6 min, respectively. Typically 1-2 µl of the PCR reaction were analyzed by agarose gel electrophoresis. PCR reaction A, carried out with forward and reverse primers binding to *CYP2D6* exon 9 and intron 2, respectively, yielded product only from duplicated *CYP2D6* genes such as **1x2, *2x2, *4x2* and **10x2*. Gene duplications such as *CYP2D6*36x2* and the *CYP2D6*36+*10* tandem did not amplify due to the presence of the exon 9 conversion. In

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contrast, a *CYP2D6*-specific forward primer binding to intron 6 (PCR reaction B) allowed amplification of all duplication arrangements regardless of their exon 9 configuration (Fig. 1). Reaction D amplified a 3.6 kb fragment and was similar to that described by Chida et al. for the detection of *CYP2D6*36x2* (Chida et al., 2002). This primer set also amplified from *CYP2D6*5*, but produced a larger, ~ 5kb long fragment.

*CYP2D6*36 genotyping:* Reaction E, a novel *CYP2D6*36* assay, contained two sets of primers. The set for *CYP2D6*36* amplified a 597 bp product only if the gene carrying the exon 9 conversion is located at the downstream position in the locus. The assay does, however, not discriminate between *CYP2D6*36* and **36x2*. The second primer set amplified a 860 bp long *CYP3A7* product as internal control for PCR performance (present in all samples). This PCR was carried out in a volume of 8 µl with JumpStart REDTaq DNA Polymerase (Sigma, St. Louis, MO). The entire reaction was separated on a 3% agarose gel containing Synergel (Diversified Biotech, Boston, MA). This assay is referred to as the '*CYP2D6*36 duplex assay'*.

Fragments F-1 and F-2 (Fig. 1) were generated from diluted PCR fragments B and C, respectively, with JumpStart REDTaq DNA Polymerase. Subsequent incubation with the restriction enzyme *Nco*I allowed the detection of the *CYP2D7* exon 9 conversion (E9-conv). *CYP2D6*-derived fragments were cut once by *Nco*I (591+184 bp) while fragment harboring the conversion remained uncut. Digestion patterns were resolved on 3% agarose gels containing Synergel. This assay is referred to as the '*CYP2D6*36 NcoI assay'*.

To detect the presence of the exon 9 conversion, the DNA of all study participants regardless of their genotype, were assayed with the '*CYP2D6*36 NcoI assay'*. All positive DNAs were re-tested and confirmed with the *CYP2D6*36 duplex assay'* on genomic DNA.

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Results

After extensive genotype analysis several subjects presented with a genotype/phenotype discordance. Notably, all four cases were African Americans. As shown in Table 2, three individuals, one adult and two neonates, had a *CYP2D6*4/*10* genotype. The neonates had been challenged with dextromethorphan on multiple occasions (coinciding with well-baby visits) indicating that the phenotype assessments truly reflected their metabolizer status. A fourth case presented with a DM/DX ratio of 0.38, just above the antimode of 0.3 at two weeks of age, and thus was initially classified as a poor metabolizer. However, the neonate assumed intermediate metabolism when re-challenged at one and two months of age suggesting that this subject's *CYP2D6*10* allele may also be compromised.

To test whether any of the four subjects carried a *CYP2D6*36x2* allele, we performed the PCR described by Chida et al. (Chida et al., 2002). Indeed, all produced a 3.6 kb long amplicon (fragment D) that suggested the presence of the exon 9 conversion in the most downstream *CYP2D6* gene within the locus (Fig. 1 and Fig. 2D). However, fragment B, a duplication-specific ~10.5 kb product that covers the entire intergenic region as well as the 'tail and head' parts of duplicated *CYP2D6* genes did not amplify in multiple attempts while it was readily generated from any other high-quality DNA that had previously been identified to carry either a *CYP2D6*1x1*, **2x2* or **4x2* gene duplication. This observation implied that the four cases did not harbor a *CYP2D6*36x2* gene duplication, but carried only one *CYP2D6*36* gene copy (Fig. 1 and Fig. 2B). *CYP2D6*36* and *CYP2D6*36x2* designations are used within this report for alleles with one and two *CYP2D6*36* gene copies, respectively. We also noted that fragment B generated from the *CYP2D6*36+*10* tandem allele was about 0.5 kb longer than the amplicon

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generated from *CYP2D6*1x1*, **2x2* or **4x2* gene duplication events (Fig. 2B). This phenomenon is currently being further characterized.

The discovery of *CYP2D6*36* in combination with another non-functional allele in phenotypic poor metabolizers demonstrated that this allele encodes a gene product that lacks appreciable activity towards dextromethorphan in vivo. To determine the frequency of *CYP2D6*36* and to examine whether any alleles other than *CYP2D6*36* harbor the exon 9 conversion, a total of 895 DNA samples (i.e. 1790 chromosomes) derived from either blood or tissue samples of Caucasian Americans, African Americans, Asian Americans and 'other or unknown' ethnicity were genotyped using the *CYP2D6*36 NcoI assay*. In addition, all DNA samples positive for *CYP2D6*10* were tested for *CYP2D6*10x2* gene duplications and *CYP2D6*36+*10* tandem arrangements by generating a ~10.5 kb amplicon (fragment B, Fig. 1 and Fig. 2B) and genotyping this fragment using a nested PCR-RFLP assay (*CYP2D6*36 NcoI assay*, product F-1 in Fig. 1 and Fig. 2). As summarized in Table 3, *CYP2D6*36* was only present in African Americans and in one Asian subject. The *CYP2D6*36+*10* tandem allele was most abundant in the Asian cohort (half of the total "*CYP2D6*10*" were tandem alleles, Table 3), but was absent in Caucasians and detected in only one African American subject and in two subjects of unknown ethnicity. In three individuals, genotyping results were compatible with either a *CYP2D6*36/*36+*10* or **36x2/*10* assignment. No *CYP2D6*10x2* or *CYP2D6*36x2* duplications were found in any subject. Interestingly, *CYP2D6*36*, **36x2* and the **36+*10* tandem arrangement were absent in the Caucasian populations tested. The frequencies of *CYP2D6*36* (including alleles that could be either *CYP2D6*36* or *CYP2D6*36x2*) were 0.53-2.5% in Africans, 2.63% in Asians and 3.33% in the 'other or unknown' group.

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We also designed and evaluated a novel assay to facilitate detection of *CYP2D6*36* and **36x2* alleles directly from genomic DNA without the need to produce a long-range PCR product. A short 597 bp amplicon was generated alongside an internal amplification control product, only when the most downstream *CYP2D6* gene contained the exon 9 conversion. This reaction was *CYP2D6*-specific and did not amplify from the *CYP2D7* gene as demonstrated by using a *CYP2D6*5/*5* DNA lacking the *CYP2D6* gene on both alleles (Fig. 1 and Fig. 2E). All DNA samples that were *CYP2D6*36*-positive with the *CYP2D6*36 NcoI assay*, were also positive with the novel assay, i.e. the '*CYP2D6*36 duplex assay'*.

The exon 9 conversion was also found in a single individual originally genotyped as *CYP2D6*2x2/*4*. As shown in Fig. 1 and Fig. 2 (lane 7), further characterization revealed that the *CYP2D6*4* allele also harbored a gene duplication and that the conversion was located on both *CYP2D6*4* gene copies (i.e., both **2x2* and **4x2* alleles must have generated fragment B since F-1 amplified thereof genotyped heterozygous for the exon 9 conversion; fragment A was derived from the *CYP2D6*2x2* allele only, since F-1 was negative for the conversion; fragment C, generated from both alleles, yielded F-2 fragments with and without the conversion; and finally, fragments D and E amplified from genomic DNA indicating an exon 9 conversion-containing gene at the downstream position). Moreover, 1846G>A, the key *CYP2D6*4* SNP, was confirmed to be present on both gene copies of the *CYP2D6*4x2* allele. This was achieved by generating a PCR fragment encompassing exon 3 through 9 with primers that specifically amplified this gene structure and using it as genotyping template for 1846G>A (or **4*) detection (not shown). The presence of this novel allele was further confirmed by pedigree analysis, which showed that both offspring of our case had inherited the exon 9 conversion-negative

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*CYP2D6*2x2* allele. This allele was designated *CYP2D6*4N* by the P450 nomenclature committee.

During the course of this study we also discovered a *CYP2D6*4* allele that lacked the C>T SNP at position 100. The subject was a *CYP2D6*4/*4* poor metabolizer who presented with a heterozygous 100C>T genotyping result (not shown). Noteworthy, 4180G>C was also absent. This polymorphism is otherwise present on all defined *CYP2D6*4* alleles except *CYP2D6*4J* according to the nomenclature web site. In addition, six other SNPs that were found on five re-sequenced *CYP2D6*4* alleles were also missing (Fig. 3). Absence of these SNPs on the novel *CYP2D6*4* variant was again confirmed by genotyping and/or re-sequencing DNA from the parents of the case. This allele has been designated *CYP2D6*4M* by the P450 nomenclature committee.

To complete the analysis of the novel alleles, the entire 6.6 kb PCR product (fragment C) from *CYP2D6*4, *4M, *4N, *10, *10* derived from a **36+*10* tandem and **36* were cloned and sequenced. The presence of SNPs and their locations in comparison to two reference sequences, AY545216 (*CYP2D6*1* reference sequence previously published, Gaedigk et al., 2005a), and M33388 are shown in Fig. 3.

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Discussion

Three cases with a poor metabolizer phenotype and an initial discordant *CYP2D6*4/*10* genotype were characterized by long-range PCR, genotyping, cloning and sequencing. Results are consistent with the presence of a 'single' *CYP2D6*36* gene in the most downstream position within the *CYP2D6* locus. The *CYP2D6*36x2* gene duplication has been characterized only in Japanese to date, and this is the first report describing an allele carrying a single *CYP2D6*36* gene copy. In addition, allele frequencies of this novel allele were determined in Caucasian, African American and Asian population samples. This discovery not only resolved the genotype to phenotype discordance in our cases, but also provided ample evidence that *CYP2D6*36* was indeed responsible for poor metabolism in vivo as previously suggested by Fukuda et al. and Chida et al. (Fukuda et al., 2000; Chida et al., 2002). Kinetic data on bufuralol 1'-hydroxylation and venlafaxine *O*-demethylation showed significantly higher Km values for *CYP2D6*36*-derived protein compared to that of *CYP2D6*1* and **10* gene products (Fukuda et al., 2000) and a *CYP2D6*21/*36x2* subject presented as a poor metabolizer towards the probe drug debrisoquine (Chida et al., 2002). The data presented here extend these findings to dextromethorphan and suggest that CYP2D6.36 protein has limited or no appreciable activity towards other drugs known to be metabolized or bioactivated through the CYP2D6 pathway.

The fourth case presented here exhibited a metabolic ratio of DM/DX of 0.38 at two weeks of age, and was by strict application of the antimode of 0.3, a poor metabolizer. This initial discordance, however, subsided with age (Table 1), suggesting that this infant had a particularly slow onset of *CYP2D6* expression compared to other subjects studied at this age. The subject's revised genotype *(CYP2D6*36/*41*) with one reduced function allele may have contributed to

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the delayed expression in this case, however, other factors than genotype may also have played a role. It should be noted that the onset of *CYP2D6* expression occurs within days after birth and was consistent with genotype at two weeks of age. The acquisition of CYP2D6 activity during the first year of life is further described and discussed in detail elsewhere (Blake et al, manuscript in preparation).

Considering the frequency of *CYP2D6*36* between 0.5% and 2.6% in African Americans and Asians, *CYP2D6*36* is one of the more common PM alleles in these populations next only to *CYP2D6*4* and *CYP2D6*5* (Nishida et al., 2000; Gaedigk et al., 2002; Ji et al., 2002a; Ji et al., 2002b). In light of our limited sample number of Asians and the absence of detailed information concerning the ethnicity of those samples (*i.e.* country of origin, degree of admixture), the frequency of *CYP2D6*36* will require further analysis in defined Asian populations. Since the *CYP2D7* exon 9 conversion has been found almost exclusively on a *CYP2D6*10* background, and Asians exhibit a high frequency of the *CYP2D6*10* allele (30-50%), it is tempting to speculate that revealing *CYP2D6*36* may, at least in part, explain the wide range of enzymatic activity observed between subjects of various *CYP2D6*10* genotypes (Kim et al., 2004).

Moreover, *CYP2D6*36* may have originated in Africa and spread to Asia, but not Europe as it has not been observed in any of our Caucasian samples, either as a single gene copy or in tandem with *CYP2D6*10* (i.e. *CYP2D6*36+*10*). This observation clearly suggests follow-up studies in African populations. To facilitate such studies and also, to make diagnostic genotyping easy and reliable, we have developed an assay that can be performed directly on genomic DNA, is specific (i.e. amplifies only from the most downstream *CYP2D6* gene within the locus) and has the potential for adaptation to high throughput genotyping platforms.

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The discovery of *CYP2D6*4M* and *CYP2D6*4Nx2* along *CYP2D6*36* underscores the highly polymorphic nature of the *CYP2D6* locus. While the presence of the exon 9 conversion on *CYP2D6*4N* is of no functional consequence (i.e. 1846G>A is the detrimental mutation causing aberrant splicing), knowledge of such gene structures is important for the accurate interpretation of genotyping results (i.e. correct allocation of SNPs or sequence variations to an allele). Likewise, absence of the 100C>T SNP on *CYP2D6*4M* does not have a functional consequence. However, the majority of *CYP2D6*4* alleles (i.e. **4A-L*) are characterized by the linkage of 100T and 1846A, and many genotyping approaches are interpreting results based on 100C/1846A linkage to assign *CYP2D6*4* and 100C/1846G (lack of the "**4*" SNP) to assign *CYP2D6*10*. Lack of 100T on a fraction of *CYP2D6*4* alleles may therefore interfere with proper allele assignment, e.g. a subject heterozygous for 100C/T and 1846G/A likely is a *CYP2D6*1/*4* with both SNPs located on the *CYP2D6*4* allele, but a *CYP2D6*4/*10* genotype is possible with 100T on *CYP2D6*10* and 1846A on *CYP2D6*4*. The presented case is our first to reveal absence of 100T on *CYP2D6*4M* as it was paired with a second 'normal' *CYP2D6*4*; in other cases however, it may have remained 'masked'. It is also the first entirely sequenced. There are only two reports in the literature describing lack of 100T on *CYP2D6*4*. In one study a single Basque individual lacked 100T on a *CYP2D6*4* allele (Fuselli et al., 2004), while 17 of 40 (40%) and 16 of 62 (26%) *CYP2D6*4* in Nicaraguans and Spanish subjects, respectively, lacked this SNP (Agundez et al., 1997). The family of our case, however, reported German and English origins and is unaware of any Spanish or Hispanic ancestors.

In conclusion, *CYP2D6*36* has been overlooked in the past and its contribution to the polymorphic expression of CYP2D6 was likely underestimated. The discovery of three poor metabolizers carrying this allele demonstrates loss of function while allele frequencies between

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0.5 and 3% emphasize its important contribution to intermediate and poor metabolism in African Americans and Asians. It is highly recommended to include *CYP2D6*36* testing for reliable phenotype prediction, especially when genotyping is performed in African Americans, subjects of African descent and Asians.

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Acknowledgement

We gratefully acknowledge the technical assistance of Liliane Ndjountché and Darren Baker. We also like to thank the participating families for their contribution.

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Footnote

Supported by R01 ES10855-05 from the National Institute of Environmental Health Sciences and in part by an intramural grant from the Katherine B. Richardson Associates Endowment Fund.

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Legends to Figures

Figure 1. Graphical display of *CYP2D6* gene arrangements.

Each line represents an allele as labeled, open boxes being genes and black boxes indicating the presence of a *CYP2D7* exon 9 conversion in a *CYP2D6* gene. Primer binding sites are given by open (*CYP2D6*-specific) and black (*CYP2D7* or exon 9 conversion-specific) circles. PCR products are labeled A to F with respective lengths as shown in kilo base pairs (kb). Fragments F-1 and F-2 are generated in nested PCR reactions using fragments B and C as templates, respectively. Note that the primers for fragment D amplify products of different lengths from *CYP2D6*36* and *CYP2D6*5* alleles. The designation of PCR fragments (A to F) correspond to the panels shown in Fig. 2.

Figure 2. Long range PCR and genotyping assays on selected DNA samples.

Panels A to F correspond to the PCR fragments shown in Fig. 1. Respective marker bands are labeled to the left and PCR product(s) in each panel to the right in kilo base pairs (kb) or base pairs (bp). Genotypes of selected samples are as indicated on the top. A *CYP2D6*5/*5* DNA in lane 8 served as control to demonstrate assay specificity (i.e. did not amplify any product with *CYP2D6*-specific primer pairs). Reactions A to E were carried out on genomic DNA, F-1 and F-2 were generated from fragments B and C, respectively. Fragment C is the 6.6 kb PCR product that is generated only from the most downstream *CYP2D6* gene and serves as our routine genotyping template. This PCR reaction was duplexed with primers generating a 3.5 kb long fragment from **1x2, *2x2* and **4x2* gene duplications (note that the *CYP2D6*36+*10* tandem did not yield the 3.5 kb 'duplication' band). M, 100 bp and 1 kb DNA ladders (New England Biolabs).

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Figure 3. Display of SNPs in resequenced *CYP2D6* allelic variants.

The two panels on the top give the SNP sequence context and the SNP coordinates on two reference sequences, AY545216 and M33388. The nomenclature numbering is based on ATG = +1 of M33388. Sequence differences between AY545216 and M33388 lead to an off-set in numbering. In the checkerboard panel open and black boxes indicate absence and presence of respective SNPs. Each line represents an allele. The line for *CYP2D6*4A* comprises sequence data for two Caucasian and three African American alleles that were identical, each other line represents a single allele. For *CYP2D6*4Nx2* and the *CYP2D6*36+*10* tandem arrangement, the sequence is derived from the most downstream gene. The bottom panel shows the location of the 5'UTR, exons (Ex), introns (In) and the 3'UTR. Amino acid changes due to non-synonymous SNP sare shown.

[1] due to PCR errors absolute number of A may vary from number shown

[2] the *CYP2D6*36* allele has an A-del at this position

[3] $P_{469}A$, $T_{470}A$, $H_{478}S$, $G_{479}R$, $F_{481}V$, $A_{482}S$

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Table 1

Primers and assay conditions for long and short range PCR reactions.

PCR Fragment [a]	Primer sequence 5’ to 3’	ann ºC [b]	Product length	Reference pattern	Variant pattern
	Long PCR on genomic DNA				
A	5’GCC ACC ATG GTG TCT TTG CTT TCC TGG 5' CCG GAT TCC AGC TGG GAA ATG CG	68	~9.5 kb		
B	5’ AGG AGG CAA GAA GGA GTG TCA GG 5' CCG GAT TCC AGC TGG GAA ATG CG	68	~10.5 kb		
C	5’ AGC CAC TCT CGT GTC GTC AGC TT 5’ CAG GCA TGA GCT AAG GCA CCC AGA C	68	3.6 kb (**36*) ~5 kb (**5*)		
	Short PCR on genomic DNA				
D	5’ AGC CAC TCT CGT GTC GTC AGC TT 5’ CGACTGAGCCCTGGGAGGTAGGTAG 5’ CACCTCTGCTAAGGGAAACAGGCC 5’ GCCAGCCTGAACATCCTTTTTGCTA	68	597 bp 860 bp	absent 860 bp	597 bp 860 bp
E	5’ ATG GCA GCT GCC ATA CAA TCC ACC TG 5’ ACT GAG CCC TGG GAG GTA GGT AG	68	6.6 kb		
	PCR-RFLP on long PCR template				
F	5' CTT CCG TGG AGT CTT GCA GG 5’ CCT GGG AGG TAG GTA GCC CTG	58	775 bp	591+184 bp *Nco*I	775 bp

[a] PCR fragments as outlined in Fig. 1; [b] ann ºC, annealing temperature

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Table 2. Description of subjects presenting with genotype-phenotype discordance.

CYP2D6 phenotype was determined from the urinary ratio of dextromethorphan to dextrorphan (DM/DX ratio) with poor metabolizer (PM) status defined as a DM/DX ratio >0.3. One subject presented as PM two weeks after birth, but acquired IM status (DM/DX <0.3>0.03 as defined by Gaedigk et al., 2003b) within two months of life. Each DM/DX ratio represents a phenotype assessment. Originally discordant and subsequently revised genotypes are presented.

Sample ID	Ethnicity	Age Group	DM/DX Ratio (Age) [a]	Original genotype (discordant)	Revised genotype (concordant)
Case 1	AA [b]	Adult	0.849	*4/*10	*4/*36
Case 2	AA	Infant	3.905 (11 d) 1.595 (34 d)	*4/*10	*4x2/*36
Case 3	AA	Infant	1.613 (15 d) 3.824 (31 d) 0.932 (65 d) 0.328 (123 d) 2.026 (185 d)	*4/*10	*4x2/*36
Case 4	AA	Infant	0.381 (14 d) 0.241 (27 d) 0.019 (64 d)	*41/*10 [c]	*41/*36 [c]

[a] Gestational age in days when phenotyping was conducted

[b] AA, African American

[c] Both genotypes are discordant with the DM/DX ratio at 14 d due to the presence of *CYP2D6*41* but are concordant with the subsequent phenotype assessments

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Table 3

Allele frequencies in Caucasians, African Americans, Asians and two study populations of mixed ethnicity. **10* denotes a 'collective' *CYP2D6*10* assignment made by testing for 100T only. Combination of additional assays (see Fig 1 and Table 2) allowed further discrimination of 'collective' *CYP2D6*10* alleles as indicated.

Allele	**CA**[a] (n=418)[b]	**AA** (n=562)	**AA-SC** (n=252)	**AS** (n=76)	**DIV Infant** (n=310) [c]	**DIV Tissue** (n=162) [d]
Total "**10*"	8	20	12	20	19	9
**10*	8	17 [e]	10	7	15	2
**10x2*	0	0	0	0	n/d [f]	0
**36*	0	3	1	1	4 (AA)	1 (AA)
**36x2*	0	0	0	0	0	0
**36+*10*	0	0	1	10	n/d	2
**36* or **36x2* [g] **10* or **36+*10*	n/a	n/a	0	1 1	n/a [h]	1 (Oth) 3 (Oth)
Frequency of **36* in % [i]	0	0.53	0.79	2.63	2.5 (AA) 0 (CA)	2.0 (AA) 3.33 (Oth)

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Table 3 (continued)

[a] CA, Caucasian American; AA, African American; AS, Asian; DIV, ethnically diverse; Oth, ethnicity other or unknown

[b] number of chromosomes genotyped

[c] ethnic diversity of infant samples (n=number of chromosomes) was CA, n=126; AA, n=160; AS, n=8, H, n=10; admixed or Oth, n=6

[d] DNA samples were derived from tissue bank: CA, n=76; AA, n=50; AS, n=2, H, n=2; NA, n=2; Oth, n=30

[e] The presence of a gene duplication could not be determined in three subjects due to lack of DNA or compromised DNA quality

[f] n/d, not determined. Not all total *"*10"* alleles could be tested for the presence of a gene duplication in this cohort due to compromised DNA quality in genomic DNA samples derived from buccal brushings) in a subset of samples

[g] Long-range PCR and diagnostic *CYP2D6*36* assays are compatible with either a *CYPD6*36/*36+*10* or **36x2/*10* genotype.

[h] n/a, not applicable

[i] Allele frequency contains *CYP2D6*36* and *CYP2D6*36* or *CYP2D6*36x2* as indicated in [g]

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Figure 1

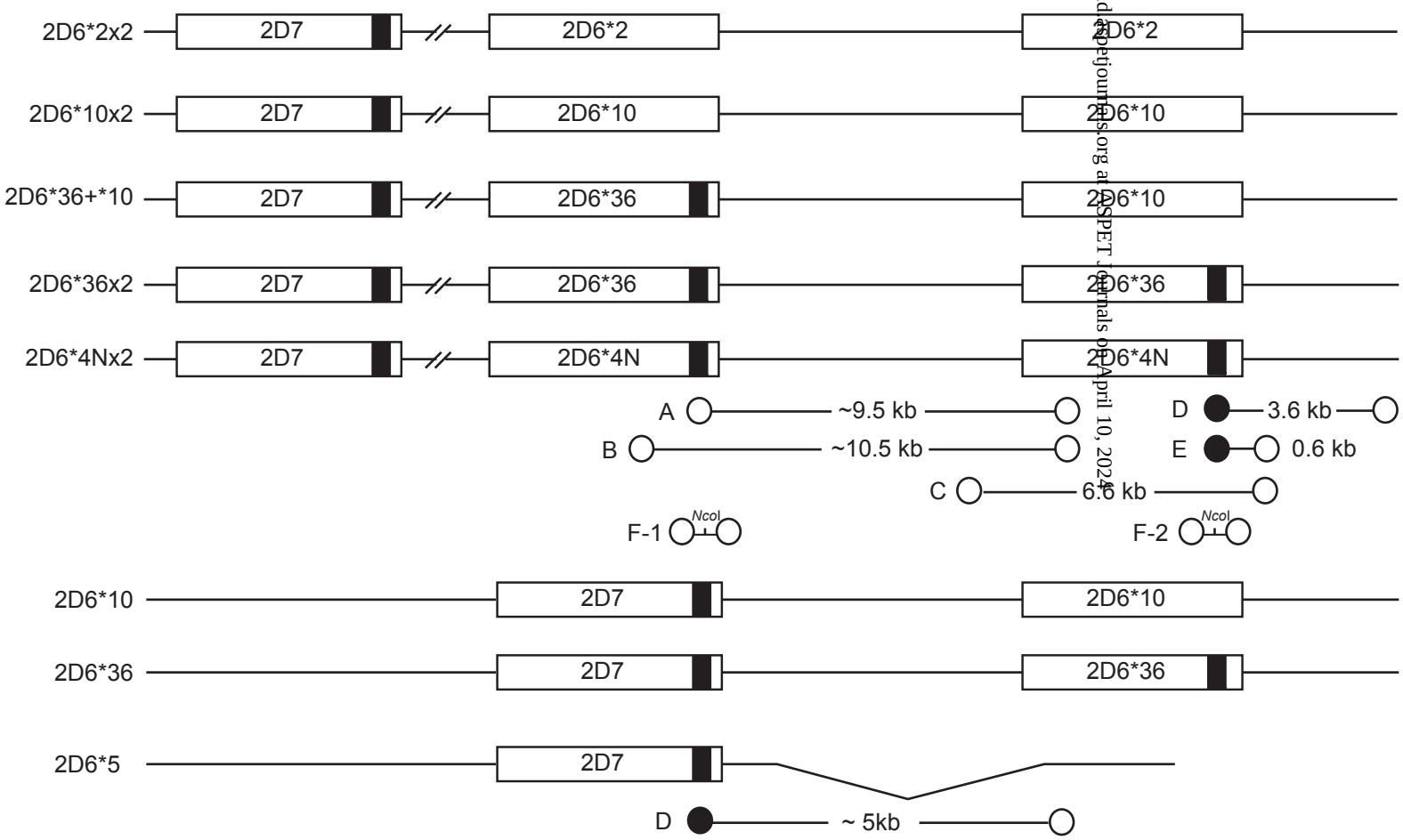

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Figure 2

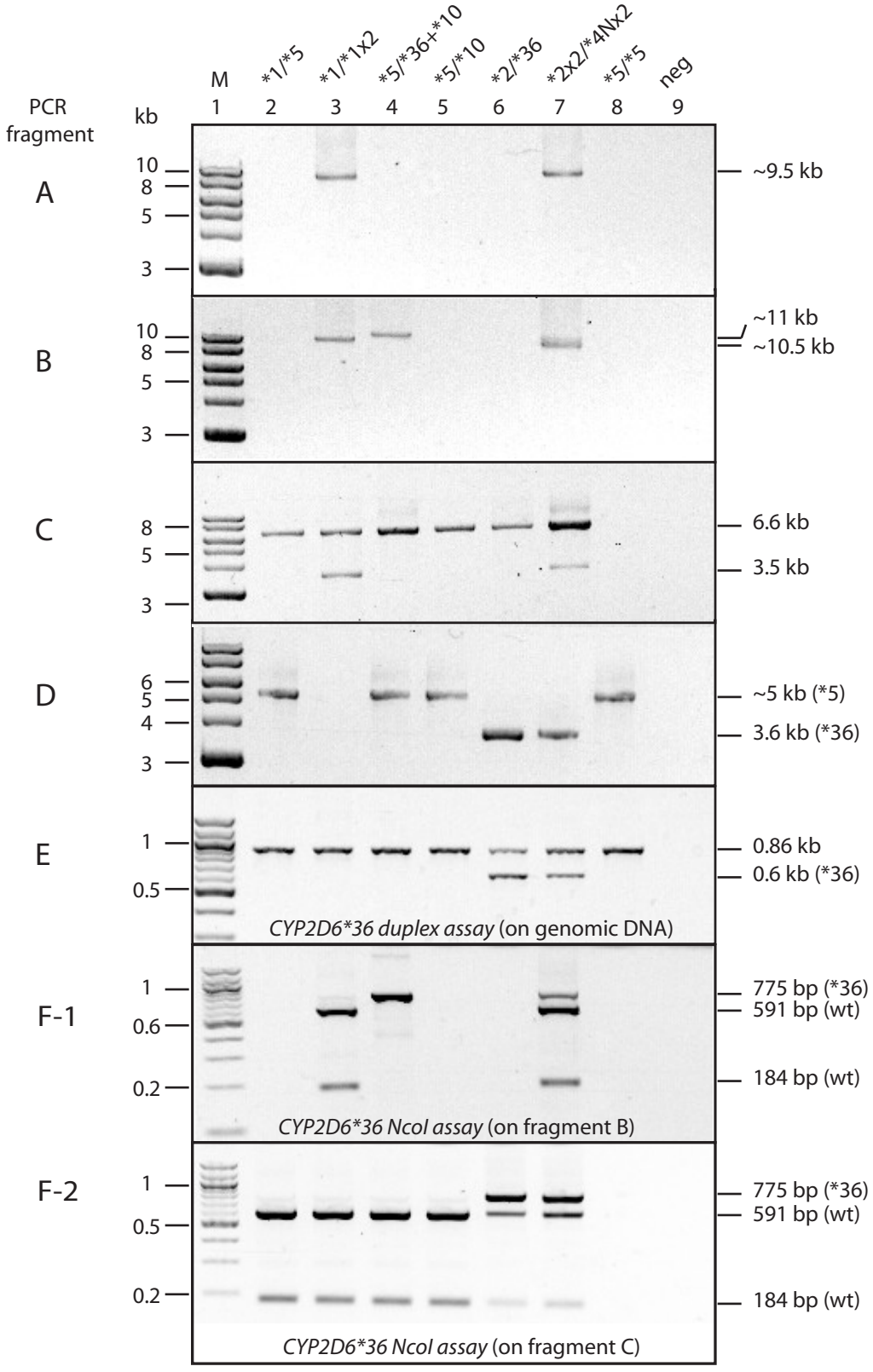

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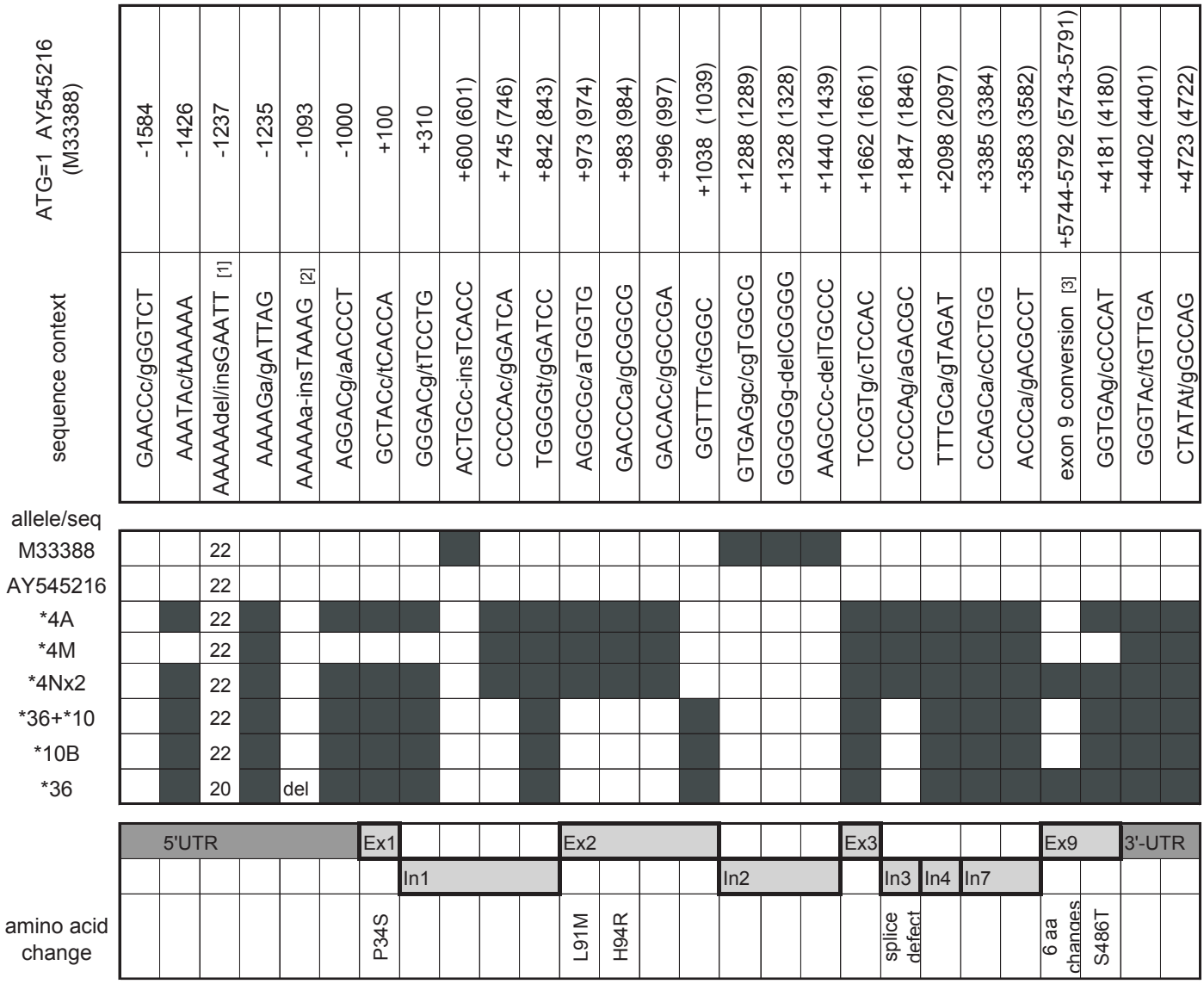
Figure 3



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