

## Carrier detection in Duchenne muscular dystrophy using molecular methods

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**Background & objectives:** Duchenne and Becker muscular dystrophies are X-linked allelic disorders which are caused by mutations in the *DMD* gene. Carrier analysis in DMD is complicated due to the heterozygous nature of the X chromosome. Several techniques have been tried for carrier analysis in families where the mutation is identified including quantitative multiplex PCR (qmPCR), Southern blot, and now multiplex ligation-dependent probe amplification (MLPA). Linkage analysis is used in cases without identifiable mutations. The present study was undertaken to determine the status of probable carriers in families where the DMD deletion/duplication has been identified for the affected index cases.

**Methods:** Carrier status was present in 150 probable carriers from 110 apparently unrelated families where the patients' mutations were known. Of these 110 families, 100 were deletions, 9 duplications and 1 point mutation. Multiplex ligation-dependent probe amplification (MLPA) was used to assess the copy number changes and direct sequencing was used for the case with the point mutation.

**Results:** Of the 150 cases, 49 were found to be carriers. Among the sporadic cases, it was observed that the rate of *de novo* mutations was very high (71%) as compared to the hereditary cases (29%), which was higher than the calculated rate (30%). It was observed that this difference was more apparent in deletion mutations than in duplications.

**Interpretation & conclusions:** Identifying the DMD carrier rates in the families with unidentified deletions and duplications and where the causative mutation could be small insertions/deletions or point mutations could throw more light into this observation. MLPA was found to be useful in detecting copy number changes in DMD carriers and this could be the method of choice for DMD carrier analysis, when the mutation is detected in the affected child.

**Key words** Carrier diagnosis - *de novo* mutations - Duchenne muscular dystrophy - multiplex ligation-dependent probe amplification (MLPA)

Majority of genetic disorders place a considerable burden on the families perpetuating the condition for the lack of effective treatment. Duchenne/Becker muscular dystrophies (D/BMD) are such lethal disorders caused by mutations in the dystrophin gene. DMD is a common paediatric neuromuscular disorder affecting 1/3500 live male births, while BMD is milder and less frequent<sup>1</sup>. The diseases are manifested with muscular weakness, hypertrophy of the calf muscles, positive Gower's sign and Pradhan's valley sign<sup>2</sup>. Due to the X-linked nature of the disorder, males carrying the mutated gene are affected, while females become carriers of the disease. Diagnosis of patients with D/BMD is usually definitive based on clinical, pathological and biochemical findings, although it is increasingly being confirmed by molecular and genetic analysis.

The first essential step in genetic counselling is to verify the diagnosis in the index case. Next, a detailed family tree is to be constructed before investigation of the possible carrier is begun. Practically, if the mother of an affected boy (proband) has another affected relative, she must be an obligate carrier. If there is an affected brother or at least one affected son, she is a possible carrier<sup>3</sup>. But in most families there is only one affected patient. Therefore, maternal female relatives of affected males are candidates for carrier assessment.

Establishing the carrier status in X-linked recessive disorders is one of the basic dilemmas in genetic counselling because female carriers are usually asymptomatic. Several biochemical and molecular methods have been used<sup>4</sup>. Essentially, in families where the proband deletion or duplication is known, dosage testing for the deleted/duplicated exons is the recommended method. For all other cases where the causative mutations could be small changes like small deletions, insertions and point mutations, gene sequencing can be attempted, though linkage analysis can be applied with a certain amount of accuracy in these families.

In this study, the carrier status of probable carriers was examined in families where the DMD gene deletion/duplication was identified for the affected index case. In addition, the usefulness of multiplex ligation-dependent probe amplification (MLPA) in carrier analysis was also studied, while also validating and comparing MLPA results with results obtained from other commonly used methods.

## Material & Methods

*Patients and samples:* The carrier status of female relatives including mother, maternal grandmother, maternal aunt, and sisters of affected males whose DNA analysis results were available and confirmed to have Duchenne / Becker muscular dystrophy was analysed. All cases were referred by the clinician from ICH&HC, Egmore, Chennai, a Government specialty pediatric hospital to Molecular Diagnostic Facility at Sundaram Medical Foundation, Chennai, for carrier diagnosis between May 2007 and July 2009. The study protocol was approved by the institutional ethical committee of Sundaram Medical Foundation. A total of 150 probable carriers were analysed from 110 apparently unrelated families. Molecular analyses done to confirm diagnosis in probands were multiplex PCR and MLPA. For one of the cases (B28), DMD gene mutation was confirmed by direct sequencing elsewhere and was found to have a frameshift point mutation (c.7348dupG) in exon 51. Carrier analysis was attempted for possible carriers in each of the families by using MLPA. Linkage by STR-(CA) segregation analysis and quantitative multiplex fluorescence PCR (qmPCR) were used to validate MLPA results on 33 samples.

Blood samples (3 ml) in EDTA were collected from female relatives suspected to be probable carriers for genetic analysis after obtaining informed consent. DNA was extracted by salting out method<sup>5</sup>, quantified and stored at -20C until tested. Multiplex PCR analysis was performed for 30 exons at the central and 5' end hot spot regions as already reported<sup>6</sup>.

*Quantitative mPCR:* Quantitative multiplex PCR<sup>7</sup> was standardized with the same conditions used for mPCR, except that the starting DNA quantity used was 250 µg/ml for all samples. Samples from male and female controls were amplified with the test samples and 12 µl of the PCR product was resolved in 2 per cent agarose gel. The resulting band was viewed in the Gel documentation system (Biorad, USA). The band intensities were analysed using the Quantity One software (Biorad, USA) and the adjusted band volumes of the test samples were compared with that of male and female controls. The results were interpreted only if the male control band volumes were half of that of the female controls.

*Quantitative multiplex fluorescence PCR (qmPCR):* QmPCR was performed for 51 exons as described by Yau *et al.*<sup>8</sup>. Two fluorescently labelled multiplex PCR assays were done to amplify 21 exons (1, 2, 3, 4, 5, 6,

7, 8, 9, 11, 12, 13, 17, 19, 21, 24, 25, 29, 30, 32 and 37) from the proximal deletion hotspot of the dystrophin gene (5' assay) and 22 exons (42, 43, 44, 45, 46, 47, 48, 49, 51, 52, 53, 54, 55, 56, 58, 59, 60, 62, 63, 68, 71 and 75) from the central deletion hotspot (3' assay). All forward primers in the assays were labelled with either fluorescent phosphoramidite fluorescein amidite 6-FAM (5' assay) or HEX hexachlorofluorescein (3' assay) (Applied Biosystems, USA). Primer sequences were obtained from Leiden Muscular Dystrophy, <http://www.dmd.nl>, accessed January 2008<sup>9</sup>.

The peak areas were then entered into the excel sheets and the dosage quotient was calculated and interpreted for deletions and duplications<sup>8</sup>.

**Multiplex ligation-dependent probe amplification (MLPA):** MLPA analysis was carried out using PO34 and PO35 probes purchased commercially from MRC, Holland (Amsterdam, Netherlands). The procedures and analysis was carried out as published earlier<sup>6</sup>.

**STR-(CA) segregation analysis:** STR-(CA) segregation analyses were performed for 11 markers spanning the DMD gene, 19n8, 3'm, 1671, 1623, i50, i49, i45, i44, 7n4, 5n4, and 5n3, according to the method described in Carsana *et al*<sup>10</sup>. using previously reported primer oligonucleotides (Leiden Muscular Dystrophy, <http://www.dmd.nl>, accessed January 2008)<sup>9</sup>. The forward primers were labelled with 5-carboxyfluorescein (FAM), PET, NED, or VIC fluorochromes. PCR products (0.5 µl from each multiplex reaction) were mixed with 0.5 µl of Gene-Scan-500 LIZ size standard (Applied Biosystems, USA) and were separated by capillary gel electrophoresis (15 kV at 60°C for 30 min) on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems) using the POP-7 polymer. The Genemapper 3.7 (Applied Biosystems) software was used for data analysis and creating a macro that allowed to label the peaks and identify the alleles of each marker automatically.

## Results

A total of 150 cases from 110 families were tested for carrier status in this study. Of the proband mutations, 100 were single or multiple exon deletions, 9 were duplications and one point mutation. Family history (more than one affected member in the family) was seen in 16 families.

**Validation of MLPA results:** MLPA results of 33 probable carriers from 23 families whose index cases showed DMD gene deletions or duplications were validated by STR-(CA) segregation analysis and

qmfPCR (Table I). STR-(CA) segregation analysis was done for 32 of the 33 cases in the study. It was seen that it was helpful in assessing the carrier status in most of the cases when it was combined with one of the direct methods. STR-(CA) segregation analysis was non-informative in six cases (17.6%). Many of the markers were homozygous for most of the cases suggesting the need to use alternative markers designed specifically for the Indian population. A representation of STR-(CA) segregation analysis results in one of the families is shown in Fig. 1.

QmfPCR analysis was done for all the 33 cases and the results were the same as that of MLPA.

**Carrier analysis results:** MLPA analysis on probable carriers revealed an average Dosage Quotients of 0.51 (range 0.34 to 0.61) for deletion and 1.46 (range 1.34 to 1.62) for duplication. Table II shows the consolidated results and carrier status of the probable carriers. Of the 150 cases, 105 were mothers, 37 sisters, six maternal aunts and two maternal grandmothers. Among the 110 families tested, 16 showed family history, 93 were sporadic cases and for one case the family history details were not available. Forty nine cases (32.7%) were tested positive for carrier status, of whom 42 were mothers (40% of all mothers) and seven were sisters of the index cases. Of the 49 cases, carrier status of 48 was confirmed by MLPA and one of the cases by direct sequencing (Fig. 2). MLPA results of one representative family each with deletion and duplication are shown in Fig. 3.

All mothers who were obligate carriers by pedigree history were tested positive for carrier status. Among the 110 families, mother's samples were not available for five and only sisters' samples were available in these cases. Of these five families, four sisters were carriers. The family where the sisters were non-carriers was not included while calculating the number of hereditary cases in the study. Therefore, of the 93 cases with sporadic forms, 27 families (29%) were tested positive for carrier status and the remaining 66 (71%) were tested negative and hence could be a *de novo* mutation in the index case or germline mosaicism in the mother.

Seven sisters to index cases (of the 37 tested) were positive for carrier status. Of these seven, two belonged to families where there was a family history of the disorder.

Inheritance of single and multi exon deletions was observed and no difference was seen among them when the complete data were taken into account. However,

**Table I.** Validation of MLPA results using CA analysis and qmfPCR

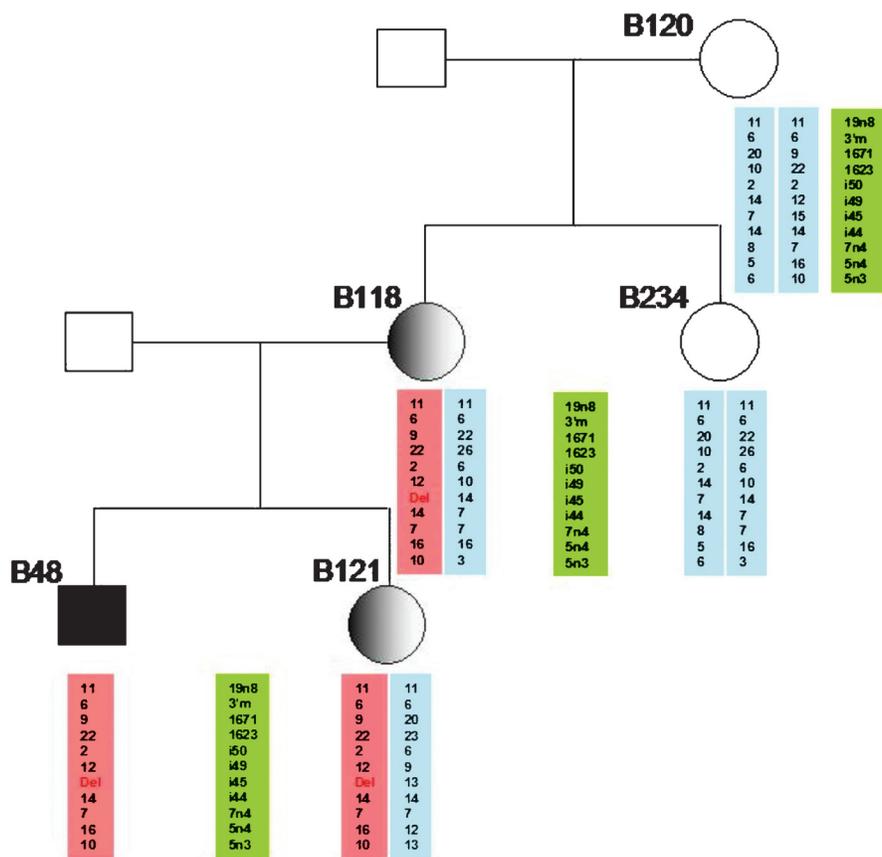
S.No.	Family No.	Carrier ID	STR-(CA) segregation analysis result	qmfPCR result	MLPA result
1	1	B118	Carrier	Carrier	Carrier
2		B120	Non carrier	Non carrier	Non carrier
3		B121	Carrier	Carrier	Carrier
4		B234	Non carrier	Non carrier	Non carrier
5	2	B190	Non carrier	Non carrier	Non carrier
6	3	B192	Carrier	Carrier	Carrier
7		B240	Non carrier	Non carrier	Non carrier
8	4	B194	Carrier	Carrier	Carrier
9	5	B206	Non carrier	Non carrier	Non carrier
10	6	B215	Non carrier	Non carrier	Non carrier
11	7	B230	Non carrier	Non carrier	Non carrier
12	8	B235	Non-informative	Carrier	Carrier
13	9	B242	Non carrier	Non carrier	Non carrier
14		B243	Non carrier	Non carrier	Non carrier
15	10	B246	Non-informative	Carrier	Carrier
16	11	B247	Non carrier	Non carrier	Non carrier
17		B248	Non carrier	Non carrier	Non carrier
18	12	B249	Non carrier	Non carrier	Non carrier
19	13	B262	Non-informative	Non carrier	Non carrier
20	14	B264	Non-informative	Non carrier	Non carrier
21	15	B267	Non-informative	Carrier	Carrier
22	16	B269	Carrier	Carrier	Carrier
23		B270	Non carrier	Non carrier	Non carrier
24	17	B275	Non carrier	Non carrier	Non carrier
25		B276	Non carrier	Non carrier	Non carrier
26	18	B278	Non carrier	Non carrier	Non carrier
27		B279	Carrier	Carrier	Carrier
28	19	B282	Carrier	Carrier	Carrier
29		B283	Carrier	Carrier	Carrier
30	20	B284	Non carrier	Non carrier	Non carrier
31	21	B86	Non-informative	Non carrier	Non carrier
32	22	B207	Not done	Non carrier	Non carrier
33	23	B312	Carrier	Carrier	Carrier

CA analysis is not informative for many cases and qmfPCR and MLPA results are the same for all the cases.

when only the mothers were taken into account, multi exon deletions showed 12% more inheritance than the single exon deletions (Table III).

Among the isolated cases, the carrier rates of deletion mutations (27%) are not significantly different

from that of duplications (50%). This may be due to the low numbers of duplication cases observed in the study and the results may show significant difference with more cases with duplication mutations added to the study (Table IV).



**Fig. 1.** Figure showing the use of STR-(CA) segregation repeat analysis for carrier detection in a family with DMD. CA-repeat microsatellite markers flanking the DMD gene locus were used for haplotyping. The affected polymorphic allele showing the deletion of intron 45 is shown in pink. In this family the proband showed exon 45 deletion of the DMD gene. It can be observed that the affected haplotype (in pink) from the mother being transmitted to both the index case his female sibling.

**Table II.** Consolidated results of all familial and sporadic cases tested in the study

Disease	Study of mothers			Status of female siblings & others probable carriers		
	Mothers	Carriers	Non-carriers	Female relatives	Carriers	Non-carriers
Familial	14	14	0	8	2	6
Sporadic	63	27	63	37	5	31
NA*	1	1	0			
Total	105	42	63	45	7	37

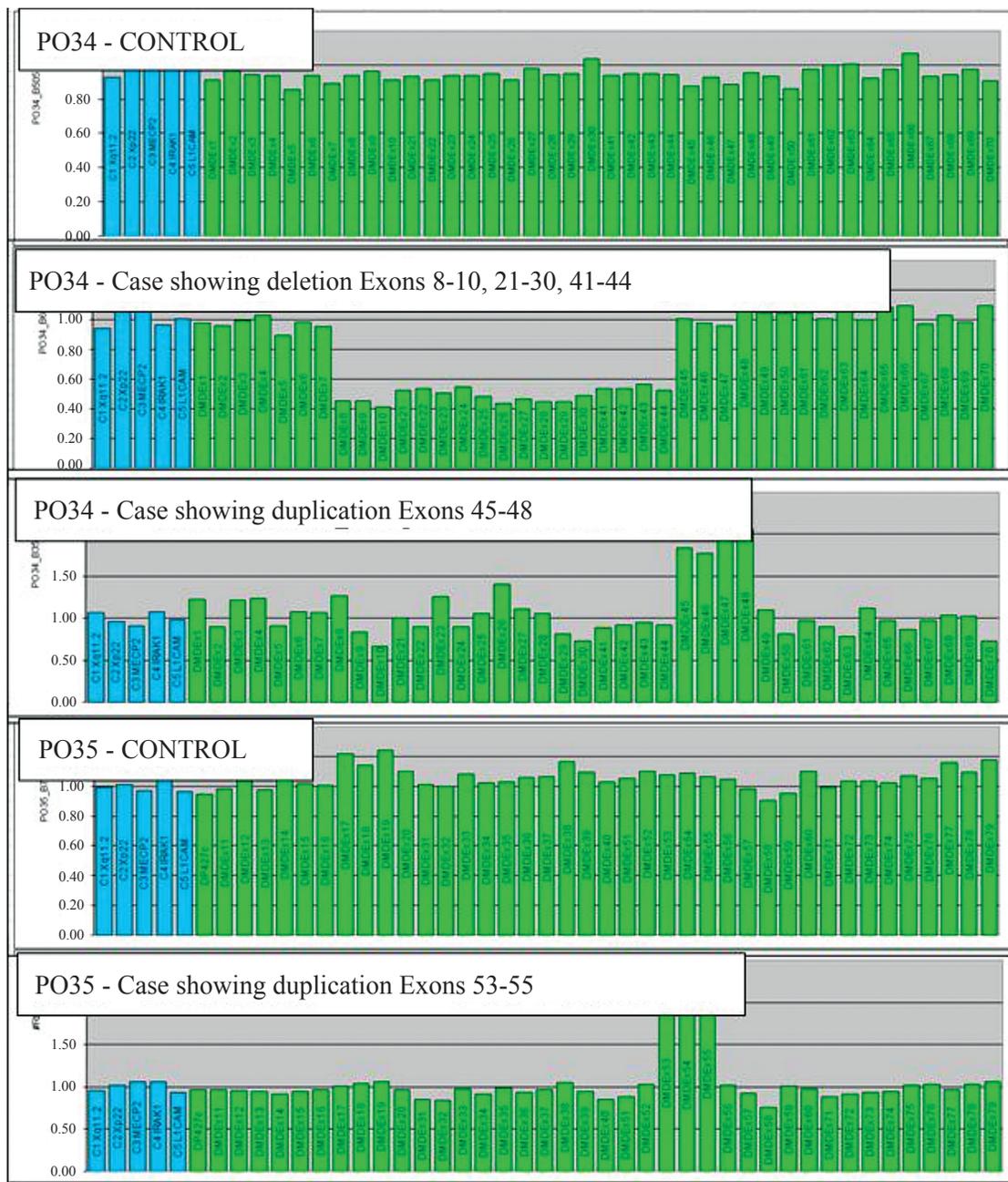
\*Details on family history were not available for one mother who showed a positive carrier status.

**Discussion**

The identification of female carriers of deletions/duplications of the *DMD* gene is a crucial point to prevent the birth of children affected by DMD or BMD. Since, theoretically, about 30 per cent of the DMD mutations are *de novo*, the risk of recurrence of the disease in

families with a single affected male is related to the carrier or non-carrier status of the mother of the patient<sup>3</sup>. Although several approaches for the identification of female carriers are available, many of these cannot be readily used for routine diagnostics. In diagnostic laboratories, DMD carrier testing has been principally





**Fig. 3.** A representative figure showing MLPA results in a normal control, proband and his carrier mother for probes PO34 and PO35. The x-axis displays the exons and the DQ is in the y-axis. Deletion can be seen as a reduction in DQ to around a value of 0.5 and in duplication DQ can be seen more than 1.5.

lower than the expected theoretical value<sup>19,20,22,23</sup>. In our study, it was observed that carrier frequency among the isolated cases was only 29 per cent and *de novo* mutations accounted for the rest of the 71 per cent. Therefore it can be seen that the occurrence of *de novo* mutations among sporadic cases of DMD was high compared to the theoretical data (30%). Our results

were consistent with other studies from India which also showed a high percentage of *de novo* mutations among DMD cases<sup>19,20</sup>. The studies from India and other earlier studies showed a low carrier frequency mainly for deletion mutations<sup>13,15-22</sup>. It has been seen that the decreased carrier rates are restricted to deletion mutations and the likelihood of being a carrier for the

**Table III.** Hereditary nature of single and multi exon deletions

Inheritance of single exon- and multi exon- deletions					
Single exon deletions			Multi exon deletions		
Overall data			Overall data		
N	Carriers	Non-carrier	n	Carriers	Non-carrier
31	12 (38.7)	19 (61.3)	107	32 (31.2)	75 (68.8)
Mothers			Mothers		
N	Carriers	Non-carrier	n	Carriers	Non-carrier
21	10 (47.6)	11 (52.4)	74	26 (35)	48 (65)

It can be noted that single exon deletions are more heritable  
Values in parentheses are percentages

other classes of mutation being in the theoretical range of 55-63 per cent<sup>21</sup>. A similar trend was observed in our study that in deletion mutations the carrier rates were 27 per cent as compared to the 50 per cent among the mothers of isolated cases with duplication mutations, which could be due to the less number of duplication cases. The mother of the isolated case with point mutation was also a carrier. Hence, the carrier risk calculations for the mother of an isolated case of DMD are not valid for deletion mutations, but appear to be valid for other mutational classes and suggest a basic biological difference in the effect of a deletion mutation compared with a non-deletion mutation<sup>21</sup>. Or it can be related to the viability of the gametes with deletion mutations as compared to the other classes of mutations<sup>21</sup>. It can also be predicted that the carrier rate in isolated cases in our study would have increased had we included the other cases who did not show a deletion or duplication mutation.

Mutation detection protocols using mPCR followed by MLPA were able to pick up mutations in 75 per cent of DMD probands. These demonstrate the importance of mutation detection protocols. Recently, MLPA assay has been suggested as the first screening test

for clinically suspected DMD/BMD patients as well as for women who have a DMD/BMD family history. However, according to the best-practice guidelines for medical genetics laboratories, it is recommended that at least two independent alternative methods are available for confirmation of each genotype<sup>24</sup>.

In conclusion, the MLPA approach seems to be a simple, rapid and reliable tool for the screening of the carrier status in cases where index case mutations are deletions or duplications of the *DMD* gene. This avoids unnecessary invasive prenatal tests with the inherent risk of miscarriage. Prenatal testing is still recommended for non-carrier mothers of an isolated male proband due to the residual chance of gonadal mosaicism. MLPA analysis could represent a method of first choice for the detection of disease-causing deletions/duplications in female relatives of affected males, especially in those cases who cannot be investigated by other approaches.

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**Table IV.** Difference in carrier rates between deletion and duplication mutations among isolated cases of DMD

n = 90	Number of isolated cases	Carrier N (%)	Non carrier N (%)
Deletion	81	22 (27)	59 (73)
Duplication	8	4 (50)*	4 (50)
point mutation	1	1 (100)	0

\*P = 0.495

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