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ORIGINAL RESEARCH

Identification of A Novel *SBF2* Frameshift Mutation in Charcot–Marie–Tooth Disease Type 4B2 Using Whole-exome Sequencing

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Abstract Charcot–Marie–Tooth disease type 4B2 with early-onset glaucoma (CMT4B2, OMIM 604563) is a genetically-heterogeneous childhood-onset neuromuscular disorder. Here, we report the case of a 15-year-old male adolescent with lower extremity weakness, gait abnormalities, foot deformities and early-onset glaucoma. Since clinical diagnosis alone was insufficient for providing pathogenetic evidence to indicate that the condition belonged to a consanguineous family, we applied whole-exome sequencing to samples from the patient, his parents and his younger brother, assuming that the patient's condition is transmitted in an autosomal recessive pattern. A frame-shift mutation, c.4571delG (P.Gly1524Glufs*42), was revealed in the CMT4B2-related gene *SBF2* (also known as *MTMR13*, MIM 607697), and this mutation was found to be homozygous in the proband and heterozygous in his parents and younger brother. Together with the results of clinical diagnosis, this case was diagnosed as CMT4B2. Our finding further demonstrates the use of whole-exome sequencing in the diagnosis and treatment of rare diseases.

Introduction

Determination of the pathogenesis of inherited diseases is a challenging endeavor because of the large number of known and unknown factors involved, although some causative factors can be revealed by traditional clinical diagnostic

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methods such as magnetic resonance imaging (MRI) [1]. To date, there are only a few effective cures for genetic diseases. For instance, some symptomatic therapies for specific diseases such as genetic-based cholestatic disorders, Wilson's disease and tyrosinemia can be cured by orthotopic liver transplantation [2].

Here, we report a case of a 15-year-old male adolescent who initially manifested weakness in both lower limbs at the age when he learned to walk, and then gradually developed obvious gait abnormalities, muscle atrophy, and talipes equinovarus, accompanied with early-onset glaucoma. The parents of the proband are consanguineous, and he has a younger brother aged 2 with normal phenotype. To further investigate this inherited disease, we performed whole-exome sequencing on the proband, his parents and younger brother, assuming that the condition was inherited in an autosomal recessive pattern.

Results

A 15-year-old male adolescent with unconfirmed Charcot–Marie–Tooth disease-like symptoms

The case in question relates to a 15-year-old male adolescent. He was born after a full-term pregnancy and caesarian section due to fetal distress with normal Apgar score, followed by normal growth and intelligence development. His consanguineous parents and the 2-year-old younger brother exhibited normal phenotype. However, he presented lower extremity weakness, gait abnormalities, foot deformities, and early-onset glaucoma, but no complaint of obvious paresthesia, numbness or pain. Biochemical examination showed that metabolites in urinary and blood samples of the probands were within normal range. In addition, his cranial MRI showed no abnormality and karyotype analyses also appeared normal. We further performed electromyography (EMG) examination on motor and sensory nerves, and found that the current nerve conduction velocity (NCV) and EMG finding is consistent with a primary demyelinating motor and sensory polyneuropathy, with moderate-to-severe slowing of conduction velocities and absence of sensory nerve action potentials (SNAPs). Besides, the clinical diagnosis mostly matches Charcot–Marie–Tooth disease type 1 (CMT1). However, mutation analysis of the main gene *PMP22* of CMT1 was negative. Therefore, the exact cause of the condition remains unclear.

CMT is a clinically and genetically heterogeneous group of inherited motor and sensory peripheral neuropathies. In general, this syndrome has an infantile or juvenile onset, with motor and sensory polyneuropathy semiology and pes cavus [3–6]. On the basis of mode of inheritance, clinical phenotypes, electrophysiological and pathological features, CMT can be classified into the following types: CMT1, CMT2, CMT4, autosomal recessive (AR)-CMT2 and dominant intermediate (DI)-CMT (Table S1). Each type can be further divided into several subtypes, depending on the underlying causative molecular defect. Based on the aforementioned EMG results and primary complaints from the patient [7], we hypothesized that this proband suffered from CMT4B2 [8–10], although idiopathic congenital talipes equinovarus [11] and other neuromuscular disorders such as spinal muscular atrophy [12] cannot be excluded.

Targeted next-generation sequencing (NGS) of the four family members

About 265,000 exonic regions from the genomic DNA of the 4 family members were captured. On an average, 12 Gb of sequencing data were generated from each individual, resulting in a mean depth of more than 110 × per base per targeted region. The coverage of the target region was over 99% (**Table 1**). In total, we identified 107,122 single-nucleotide polymorphisms (SNPs) from the exome of the proband (**Table 2**) by using SOAPsnp based on Bayes' theorem, by filtering out low-quality mutations according to the criteria (reads < 20, depths < 4, estimated copy number > 2 and the interval in between < 5). Numbers and distribution of SNPs and insertion-deletions (indels) of the four family members are listed in Table 2 and **Table 3**, respectively. Next, we filtered the SNPs and indels according to the filtering strategy as delineated in **Figure 1**. Based on the initial screening criteria with variant location and frequency ⩽ 0.005 in dbSNP, Hapmap and 1000 Genomes Database, and mutation type, we identified 3592 SNPs and indels. The subsequent restrictive filtering control further narrowed down the list and only 542 SNPs and indels were retained.

Identification of a novel *SBF2* mutation, c.4571delG, in CMT4B2

Based on the family history and clinical results (**Figure 2A**), we determined that the condition is transmitted in an autosomal recessive pattern. As a result, only homozygous variations identified in the patient were retained for further analysis, since heterozygous variations carried by the parents demonstrated normal phenotype. We therefore obtained 32 SNPs and indels from 29 genes (Table S2). Among them, only the gene *SBF2* was found to be related to the phenotype.

SBF2 (also called *MTMR13* or *DENND7B*, MIM 607697) is located on chromosome 11, base pairs 9,800,213–10,315,753.

Table 1 Exon coverage and depth using whole-exome sequencing for the four family members

Exome capture statistics	Father	Mother	Son-case	Son-control
Target region (bp)	44,234,141	44,131,461	44,234,141	44,234,141
Raw reads	132,038,330	143,078,102	123,878,094	156,397,584
Raw data yield (Mb)	11,883	12,877	11,149	14,076
Mean depth of target region (×)	126.08	136.35	115.18	145.29
Coverage of target region (%)	99.40	99.41	99.41	99.05
CCDS exons (read coverage)	265,204	264,392	265,247	264,808
CCDS genes (read coverage)	18,386	18,335	18,386	18,381

Note: CCDS, Consensus Coding Sequence Dataset.

Table 2 Detailed whole-exome sequencing data for SNPs detected in the four family members

Type	Father	Mother	Son-case	Son-control
Total number of SNPs from each member	112,238	111,822	107,122	111,669
Missense	13,246	13,221	12,748	12,748
Readthrough	92	88	86	85
Nonsense	177	155	158	157
Splice site	2798	2732	2647	2731
Synonymous-coding	10,876	10,763	10,318	10,386
5′-UTR	9211	9055	8741	9040
3′-UTR	3387	3421	3238	3425
Intronic	70,013	69,952	66,830	70,645
Intergenic	2438	2435	2356	2452
Homozygous	45,121	44,639	49,047	48,643
Heterozygous	67,117	67,183	58,075	63,026
Total number of SNPs from all members				154,336

It contains 40 exons and spans over 600 kb. *SBF2* encodes SET binding factor 2, a novel inactive phosphatase of the myotubularin family [8,13], which is present in various tissues such as the cord and the peripheral nerve. Mutations in *SBF2* may cause CMT with early-onset glaucoma (CMT4B2) [7]. In humans, myotubularin-related proteins (MTMRs) constitute a large family with 14 members and have been suggested to work in phosphoinositide-mediated signaling events that may also convey the control of myelination [8]. Recessive mutations in *SBF2* are typically found in patients with redundant myelin loops or myelin outfoldings in sural nerve biopsies [8]. Remarkably, our sequencing data revealed one novel mutation, c.4571delG, in exon 34 of the *SBF2* gene, which was detected as homozygous in the proband and heterozygous in the other three family members. This was also validated by the Sanger sequencing method (**Figure 2B**). The c.4571delG (p.Gly1524Glufs*42) of *SBF2* is a frameshift deletion mutation that causes a mutation in the myotubularin-like phosphatase domain of the protein. The deletion of a single guanine residue at codon 1524 within exon 34 produces a frameshift mutation, which generates a stop codon at codon 1566 (p.Gly1524Glufs*42) and results in a premature termination codon. The parents carried this mutation in heterozygous form and were considered obligate carriers. The proband harbored the c.4571_4571delG in the homozygous form, while his younger brother with normal phenotype carried this mutation in its heterozygous form. The same mutation is also inherited from one of their consanguineous parents. The mutation was not present in the dbSNP and 1000 Genomes Database.

The catalytically-inactive SBF2 associates with MTMR2, which is an active lipid phosphatase also belonging to the MTMR family. MTMR2 is found in the mutated form in CMT4B1 [9,10]. Previous studies revealed a possible scenario in which MTMR phosphatases play roles in regulating endosomal trafficking of phosphatidylinositol 3-phosphate (PI(3)P) and bisphosphate PI(3,5)P2 [11,12]. However, molecular and cellular mechanisms addressing the correlation between the catalytic activity of MTMR phosphatases and their mutation in CMT4B remain to be elucidated. Our study showed that the c.4571delG mutation generated a truncated SBF2 protein (**Figure 2C**), which may disrupt the soluble and membrane-associated protein complex formed by SBF2 and MTMR2, leading to defects in PI(3)P and PI(3,5)P2 regulation. Because this mutation causes a premature termination, the production and function of the protein would be altered and such alteration was inherited from the consanguineous parents who carried the heterozygous deletion mutation but exhibited normal phenotype. Therefore, we considered the homozygous deletion mutation to be "pathogenic". However, to verify this possibility, further experimental evidence would be required.

Table 3 Detailed whole-exome sequencing data for indels detected in the four family members

Type	Father	Mother	Son-case	Son-control
Total number of indels from each member	7174	7073	7024	7352
Frameshift	308	309	289	260
CDS-indel	184	175	183	212
Splice site	410	387	380	421
5′-UTR	664	631	642	637
3′-UTR	256	228	247	264
Intron	5233	5221	5149	5440
Promoter	35	38	33	35
Intergenic	84	84	101	83
Homozygous	2761	2570	2863	3275
Heterozygous	4413	4503	4161	4077
Total insertions	3223	3218	3220	3542
Total deletions	3951	3855	3804	3810
Insertion-coding	208	217	206	229
Deletion-coding	284	267	266	243
Total number of indels from all members				11,502

	Father	Mother	Son-case	Son-control	Total
SNP	112,238	111,822	107,122	111,669	154,336
Indel	7174	7073	7024	7352	11,502

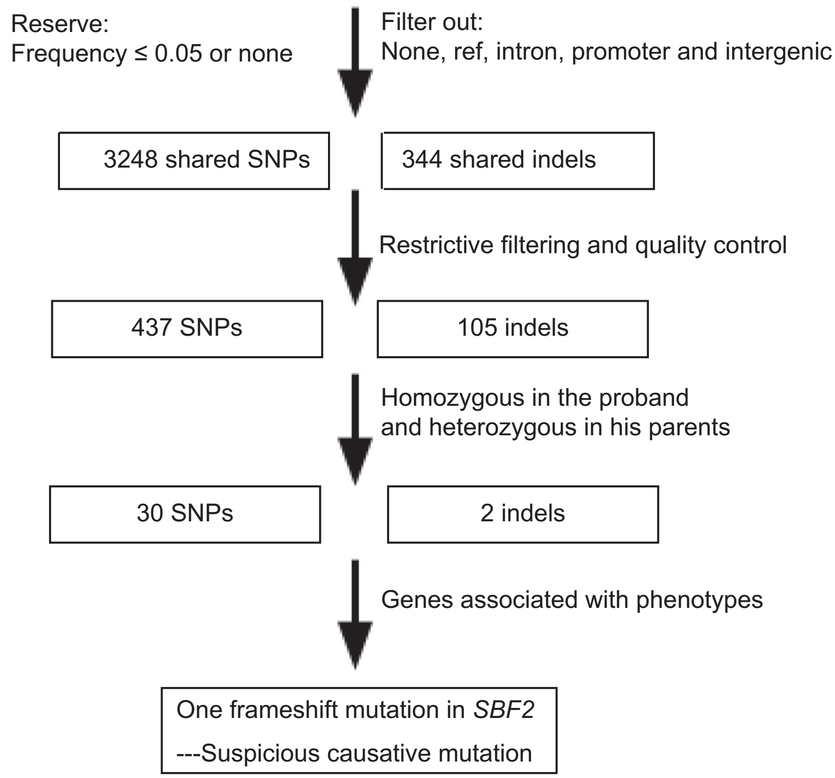

Figure 1 Bioinformatic analysis pipeline for whole-exome sequencing data in the current study
The mutations detected by the whole-exome sequencing were filtered through a series of steps. First, the low-quality mutations were filtered out (criteria: reads < 20, depths < 4, estimated copy number > 2 and the interval in between < 5). Second, the SNPs and indels were filtered based on the frequency and location mutation type. Third, candidate SNPs and indels were further filtered considering the database frequency and mutation types by adopting the pedigree analysis procedures developed in house (only 542 SNPs and indels were retained). Finally, we took into account the inheritance mode and phenotype and the candidate gene *SBF2* was found to be related to the phenotype. None in SNP category indicate that the mutation was detected in the SOAPsnp but was filtered out at the first step, whereas none in indel category indicate lack of indel in the tested sample. Ref indicates identity with the reference sequence. Intergenic, intron and promoter indicate that SNP or indel is located in the intergenic region, intron or promoter region, respectively.

Discussion

Here we describe a novel mutation of the CMT-associated gene *SBF2* in a Chinese patient. CMT is a clinically and genetically heterogeneous group of inherited motor and sensory peripheral neuropathies, with a prevalence of 17–40 per 100,000 inhabitants [14,15]. In general, this syndrome has an infantile or juvenile onset, with motor and sensory polyneuropathy semiology and pes cavus [3–6]. Mendelian segregation in families may follow either autosomal dominant, autosomal recessive or X-linked patterns. Many previous studies have shown that molecular genetics can be used in the typing of CMTs such as autosomal dominant and X-linked inheritance [16]. It is now used in case of rarer types of CMTs, such as CMT4 with autosomal recessive pattern, that are similar to CMT1 with dominant forms, but are usually more severe with an earlier onset age [17,18]. According to autosomal recessive inheritance and both axonal and demyelinating neuropathies, CMT4 can be differentiated from other forms of CMT. Nerve conduction velocities in these individuals are often below 40 m/s. The affected individuals exhibit the typical CMT phenotype of distal muscle weakness and atrophy associated with sensory loss, and frequently, pes cavus foot deformity. These individuals tend to present these symptoms at earlier ages and with more severe disease progression, although many CMT4 subtypes exist with extensive clinical overlap [13,19–21].

Up till now, 9 genes associated with CMT4 subtypes have been discovered: *GDAP1* (CMT4A), *MTMR2* (CMT4B1), *SBF2* (CMT4B2), *SH3TC2* (CMT4C), *NDRG1* (CMT4D), *EGR2* (CMT4E), *PRX* (CMT4F), *FGD4* (CMT4H) and *FIG4* (CMT4J) [22]. These subtypes can be differentiated through clinical features, ethnic background and neuropathological features. However, most of these subtypes can only be differentiated through genetic tests. For instance, CMT4B1 is caused by mutations in the gene encoding MTMR2 on chromosome 11q22 [23–25], and mutations in the *SBF2* gene have

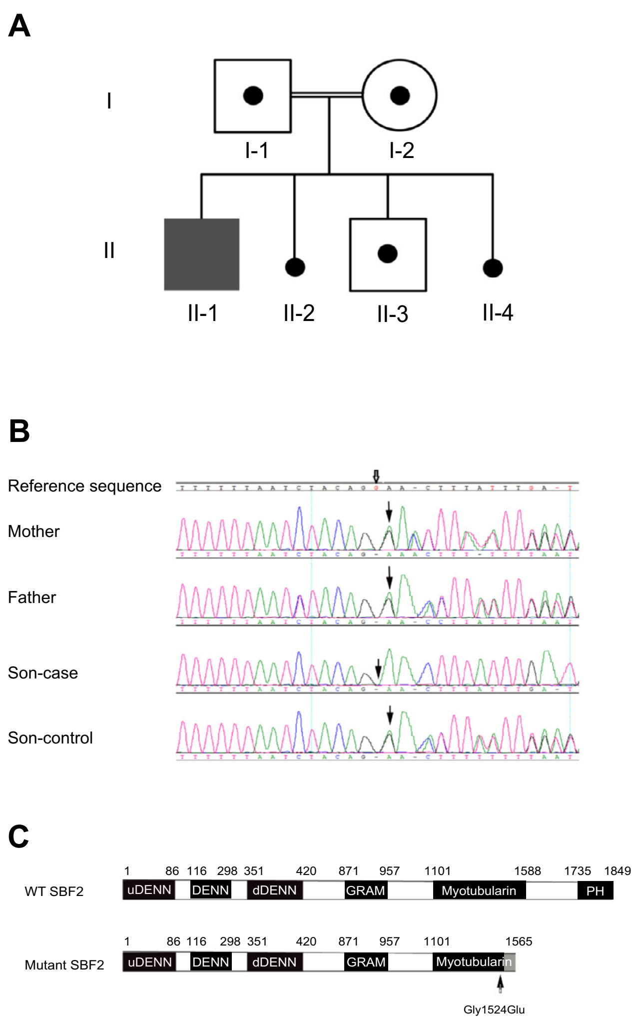

Figure 2 Pedigree diagram of the family
A. The proband (II-1, Son-case) showed clinical manifestations, but not his consanguineous parents (I-1, Father; I-2, Mother) and younger brother (II-3, Son-control). II-2 and II-4 were subjected to active abortion. **B.** Diagonal arrow indicates the *SBF2* c. 4571del; the proband carried c. 4571delG, a 1-bp deletion in exon 34 of *SBF2* in homozygous form; his parents and younger brother carried this mutation in the heterozygous form. The GenBank accession number for the reference sequence is NG_008074.1. **C.** Schematic representation of wild type and the potential mutated SBF2 protein. The c. 4571delG deletion causes amino acid change at codon 1524 (Gly1524Glu), leads to the frame shift onward and thus creates a stop codon at codon 1566 (p.Gly1524Glufs*42). This premature termination may result in a truncated SBF2 protein product comprising 1565 amino acid residues or lack of expression due to the nonsense-mediated decay. The gray box represents the 42 C-terminal missense-mutated residues including the Gly1524-Glu mutation. DENN, differentially expressed in neoplastic versus normal cells; uDENN, upstream DENN; dDENN, downstream DENN. Myotubularin indicates the myotubularin-like phosphatase domain. PH, pleckstrin homology domain.

been identified in CMT4B2 on chromosome 11p15 [8,26]. At present, there are still no effective cures for CMT4; however, we can administer symptomatic treatments by the neurologist, physiatrist and orthopedic surgeon, such as special shoes for foot drop correction and walking assistance, surgery for severe pes cavus, exercise for recovery, and symptomatic treatments for pain, depression and other symptoms [4,27–29].

In this study, we detected 32 homozygous mutations in 29 genes in a patient with unconfirmed CMT disease-like symptoms and the homozygous deletion mutation c.4571delG in CMT4B2-related gene SBF2 was considered pathogenic. Our protein domain organization analysis suggests that the c.4571delG mutation introduces a premature stop codon, which, if homozygous, may result in the loss of gene expression due to the nonsense-mediated decay or production of a truncated SBF2 protein lacking pleckstrin homology (PH) domain. The PH domain is present in more than 100 cellular signaling proteins, *e.g.*, protein kinases and GTPases, and characteristically binds to proteins such as phosphatidylinositol within biological membranes [30]. It plays multiple roles in navigating proteins to different types of host membranes and recruiting them to appropriate subcellular compartments. In addition, PH domains are believed to bind to the heterotrimeric Gβγ protein complex: G protein, protein kinase C and small GTPases [31]. However, it appears that PH domains, despite sharing low-sequence homology and non-conserved protein function, have a conserved core structurally composed of seven β-strands and one C-terminal α-helix [32]. Here, our finding of the *SBF2* c.4571delG mutant may introduce the possibility of disruption or dysfunction of SBF2-MTMR2 complex formation, which may, in turn, alter PI(3)P and PI(3,5)P2 regulation due to the PH domain deletion. Several studies also showed that *SBF2* was identified as the disease-causing gene of CMT4B1 and CMT4B2 by molecular cloning [8,13,33]. Nonsense or splice-site mutations affecting the N-terminus part of the SBF2 protein have been identified in patients with CMT4B2 [13,34]. Irregular myelin structure (outfolding) is a characteristic sign of CMT4B2, and the mutations reported in this study may result in nonfunctional proteins. In addition, studies found that mutations of *SBF2* can lead to thrombocytopenia [35], pancreatic adenocarcinoma [36] and hereditary neuropathies [8] as well.

NGS has significantly facilitated genome analysis. Whole-exome genome sequencing (WES) of all protein-coding DNA is well justified as an efficient strategy to search for alleles underlying rare Mendelian disorders [19]. Protein-coding genes constitute only approximately 1% of the human genome, but harbor ~85% genetic mutations that determine disease-related traits. In contrast to the laborious approach of SNP homozygosity mapping, the exome sequencing approach is more time-saving, is independent from shared allelic heritage and can be done in the presence of allelic heterogeneity [37]. When WES was initially introduced, its utility was highly debated, with respect to high cost, incomplete coverage of exome and low sensitivity for structural variation. However, WES will likely be employed much more commonly in the future because of the practical advantages of the technology [38]. In addition to being applied in the diagnosis of Mendelian disorders, WES can also be used in the analysis of complex diseases, such as Parkinson's disease, hypertension and autism, and will be helpful for individualized medical treatments [39].

In summary, we found a novel frameshift mutation, c.4571delG(P. Gly1524Glufs*42), in the *SBF2* gene, which likely leads to the CMT4B2 in the proband. Furthermore, our study shows that whole-exome sequencing can provide fast and accurate sequencing information for clinic diagnosis. Therefore, when a genetic disorder is strongly suspected, even in the case when traditional clinical genetic testing has difficulty in making a definite diagnosis, the application of NGS should be considered. This approach, together with continuing improvement of sequencing technology, allows medical genomics to be a routine diagnostic method for rare Mendelian disorders in the near future.

Materials and methods

Blood samples

According to the institutional ethical procedures, the peripheral blood samples of the proband, his parents and younger brother were obtained with their informed consents.

DNA extraction

Genomic DNA was extracted from 200 μl of peripheral venous blood by standard procedures using Qiamp Blood DNA mini Kit (Qiagen, Hilden, Germany) as instructed by the manufacturer. DNA integrity was evaluated by 2% agarose gel electrophoresis. All DNA samples were stored at −20 °C after analysis with NanoDrop.

Whole-exome sequencing

Whole-exome sequencing was carried out using the SeqCap EZ Human Exome Library v3.0 (64 M). Prior to sequencing, 3 μg of qualified genomic DNA from each sample was randomly fragmented to 200–300 bp in size on a Covaris Acoustic System, followed by end-repair, A-tailing and pair-end index adapter ligation. The adapter-ligated templates were purified using AgencourtAM Pure SPRI beads and amplified by 4 cycles of ligation-mediated PCR (LM-PCR): 2 min at 94 °C; 4 cycles of 10 s at 94 °C, 30 s at 62 °C and 30 s at 72 °C; followed by 5 min extension at 72 °C. LM-PCR products were hybridized to the SeqCap EZ Oligo pool for enrichment. After the hybridized fragments were bound to the streptavidin beads in 24 h, non-hybridized fragments were removed. Next, captured LM-PCR products were amplified by PCR (2 min at 98 °C; 15 cycles of 10 s at 98 °C, 30 s at 60 °C and 30 s at 72 °C; then 5 min at 72 °C), and analyzed using the Agilent 2100 Bioanalyzer and quantitative PCR to estimate the enrichment magnitude. The captured library was sequenced using Illumina HiSeq2000 Analyzers. We conducted 90 cycles per read to generate paired-end reads and 8 bp of the index tag following standard sequencing protocols from the manufacturer.

Sanger sequencing

Mutations identified by whole-exome sequencing were validated by Sanger sequencing. Primers flanking the candidate loci were designed based on reference genomic sequences of the Human Genome from GenBank in NCBI [GenBank ID: reference GRCh37(hg19)] and were synthesized by Invitrogen (Shanghai, China). PCR amplification was carried out using ABI 9700 Thermal Cycler with the primers (sense 5′-GGACTCCTCTTGTGCATTCTG-3′ and antisense 5′-GATAGACTGCTGGCTGCTTAG-3′). Subsequently, all PCR products were sequenced on ABI PRISM 3730 automated sequencer (Applied Biosystems). Genomic sequence data analysis was performed by DNASTAR SeqMan.

Authors' contributions

MQ, XY and MC conceived and designed the experiments. YC performed the experiments and analyzed the data. YC, HH and NL contributed reagents/materials/analysis tools. MC, JW, LT, JW, WW wrote the paper. MQ, XY, HH, MC, HC and TW revised the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have declared that no competing interests exist.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gpb.2014.09.003.

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