DYNAMIC QUANTITATIVE TRAIT LOCI AND COPY NUMBER VARIATION: THE MISSING HERITABILITY OF COMPLEX AGRONOMIC TRAITS

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ABSTRACT
There is a great need for improved crop varieties to feed the growing population under the changing climate. To achieve this, a central goal is to map genotype to phenotype. Genetic studies have identified thousands of loci controlling various agronomic traits, revealing important biological pathways and providing valuable insights into genetic basis of many traits variation. However, genome-wide association studies (GWAs) have explained relatively a small heritability of most complex trait. This has led to an important question of where is this 'missing' heritability. This study examined the potential sources of the 'missing' heritability and provides some experimental data that offer ideas on the underlying genetic architecture of complex trait such as biomass accumulation in maize. The study used 12 main effect and 6-pair of epistasis quantitative trait loci **(**QTL) displaying different patterns of expression at different developmental time point in 261 maize genotypes. Genotyping of the 261 genotypes was carried out using MaizeSNP50 chip. In addition, copy number variation (CNV) and presence absence variation (PAV) was used to study the genetic architecture of maize genome in 34 maize genotypes. The identified dynamic QTL and CNV were mapped on maize B73 as reference genome. A total of 182 genes were found to be harboured in the detected QTL regions. A complex CNV architecture, such as smaller CNV nested within larger CNV or overlapping CNV regions was detected throughout the maize genome, which may explain the extraordinary traits variation observed in maize. The complex CNV genomic architecture, differential gene expression and their interactions at different developmental time points, which are missing in many GWAs, may explain the missing heritability. Consequently, the genetic model from final trait values cannot reflect the real gene action during the entire growth and development of the plant. Therefore, it is necessary to understand the CNV and the dynamics of gene expression for complex trait at different developmental stages as a basis for quantitative trait manipulation.
Keywords: Copy Number Variation; Complex Trait; Heritability; Gene Action; Genome-Wide Association Studies

INTRODUCTION
The major goal of plant geneticists is to understand how genetic variation contributes to phenotypic variation in the population. The genetic sources of phenotypic variation have been a major focus in plant breeding studies aimed at identifying the causes of trait variation, improving agriculture productivity and understanding adaptive processes. Many agronomic traits are complex and controlled by many genes, each with small additive effects (Bernardo, 2008; Zuo and Li, 2014). Genome-wide association (GWAs) studies hold great promise for the dissection of complex traits (Yu and Buckler, 2006; Stich and Melchinger, 2010). The approach provides a high-resolution method for mapping quantitative trait loci (QTL) (to the gene level) based on linkage disequilibrium (Yu and Buckler, 2006). Many genetic variants contributing to complex traits have been identified (Bian *et al*., 2013; Busemeyer *et al*., 2013; Liu *et al*., 2014; Würschum *et al*., 2014; Bullucci *et al*., 2015; Bac-Molenaar *et al*., 2015). When several genes have been linked to a trait, both the individual and cumulative effects are small and not enough to explain estimated heritability.

In maize, QTL analysis has been strongly supported through sequencing and assembly of the reference genome (Schnable *et al*., 2009) and derived genotyping approaches (Ganal *et al*., 2011) and has been applied to a wide variety of morphological and physiological traits (Hao *et al.,* 2011; Zheng and Liu 2013). However, they explain only a few percent of the phenotypic diversity, hence the question; 'where is the missing heritability?' (Manolio *et al.,* 2009; Eichler *et al*., 2010). These and the vast majority of other QTL studies in plants assess the expression of traits at a certain stage, frequently at final harvest (Buckler *et al*. 2009). Very limited information has, therefore, been reported on dynamically acting genetic factors in plants accessed via monitoring trait expression at multiple time points (Bian *et al.,* 2013; Busemeyer *et al*., 2013; Liu *et al*., 2014; Würschum *et al*., 2014; Bullucci *et al*., 2015; Bac-Molenaar *et al*., 2015). Moreover, structural variation has been recognized as a major contributor to genomic diversity in various organisms (Henrichsen *et al*., 2009; Diskin *et al*. 2009; Springer *et al*. 2009; Conrad *et al*., 2010; Belo' *et al*., 2010; Yu *et al*., 2011)). Maize genomes are rich in structural diversity, including copy number variation (CNV) and presence absence variation (PAV), but this type

of variation is still poorly understood (Springer *et al*., 2009; Belo' *et al*., 2010; Swanson-Wagner *et al*., 2010). Copy number variation (CNV) describes DNA sequences (usually considered to be larger than 1 kb in size) that are present in genomes being compared albeit in different numbers of copies (Springer *et al*., 2009). The most extreme form of CNV is PAV, which refers to sequences that are present in some genomes but missing in others (Springer *et al*., 2009; Swanson-Wagner *et al*., 2010). Recent reports have suggested a role of CNV, either individually or in aggregate, as the cause of unexplained genetic variation (Springer *et al*., 2009; Diskin *et al*., 2009).

The inability to find some genes has several possible answers. The rare variants are detectable only when sample size is adequate at the local level (Manolio *et al*., 2009; Eichler *et al*., 2010; Luo *et al*., 2011). In addition, in many populations allelic heterogeneity of same gene exists and these are associated with different phenotypes (Bergelson and Roux 2010; Wood *et al*., 2011; Zhang *et al*., 2011). The single-marker linkage is also affected by genetic heterogeneity, especially, when multiple major loci are involved and in linkage disequilibrium (LD) with each other (Platt *et al*., 2010). The epistatic interactions variations normally go undetected because epistasis can only be determined by sequential genome-wide scan of major loci (Storey *et al*., 2005). The epigenetic variation is also a likely source of missing heritability (Johannes *et al*., 2009). This paper tries to advance two additional possible answers to missing heritability, i.e., the complex CNV/PAV genome architecture and dynamically acting genetic factors in plants. The information on these two genetic variants is limited or lacking. The paper also tries to highlight some of the constraint that maybe faced in an attempt to incorporate of CNV/PAV and dynamic genetic factors (dynamic QTL) in GWAs.

MATERIALS AND METHODS

Copy number variation (CNV) and presence absence of variation (PAV)

A 2.1 M oligonucleotide NimbleGen microarray designed by Roche NimbleGen (Springer *et al*., 2009) was used in this experiment. Array comparative genomic hybridisation (aCGH) was conducted according to NimbleGen aCGH analysis protocol, using 34 maize inbred lines and B73 as reference genome. Genomic DNA (gDNA) was isolated from leaf tissue of 2-weeks old maize seedling (10 plants per inbred line) using a modified CTAB protocol (Mace *et al*., 2003). Equal amounts of DNA were pooled from the ten individuals per inbred line to constitute the working gDNA samples. Test (inbred lines) and reference (B73) gDNAs (1µg each) were labeled separately with fluorescent dyes, Cy5 and Cy3 respectively, using NimbleGen dual-colour labelling kit. Labeled gDNAs were then combined and hybridized to the microarrays for 72 hours in a NimbleGen hybridization station at 42° C. The hybridised arrays were scanned at 2µm resolution with an Agilent scanner and images were extracted and analyzed with NimbleScan v2.6 software. For each test inbred line, two genome-wide aCGH profiles were obtained, representing the log2-ratios of measured fluorescent intensities for test inbred line vs. B73. All aCGH profiles were normalized and were then analyzed by a three-state Hidden Markov Model (HMM) described in Seifert *et al* (2012) to identify CNV and/or PAV between a test inbred line and the B73 reference genome. Sigmal-map was used to map CNV and/or PAV to their genomic locations. This work was carried out in genomic centre laboratories, Leibniz-Institute of Plant Genetics and Crop Plant Research (IPK-Gaterseleben), Germany.

Dynamically acting genetic factors (Dynamic quantitative trait loci)

Phenotyping

A panel of 261 inbred lines was phenotyped using an automated high-throughput phenotyping system (LemnaTec scanalyzer 3D) for their biomass accumulation from 8 – 42 days after sowing (DAS) in three seasons (2011-2012). The experiment was laid out in an incomplete randomised block design and replicated twelve times. Plants were imaged every day from 8 - 42 DAS. The estimates of fresh shoot biomass were extracted from the digital images taken daily during the growth period (8 - 42 DAS). The Integrated Analysis Platform (Klukas *et al*., 2014) was used to calculate plant biomass volume from images acquired daily as estimates of biomass accumulation during the plant growth period.

Genotyping

The 261 maize inbred lines were genotyped using the Illumina SNP chip MaizeSNP50 containing 56,110 evenly spaced SNPs distributes across the ten maize chromosomes (Ganal *et al*., 2011). Maize is a diploid with ten pairs of chromosomes (n = 10). A total of 35,682 loci were used in this study after filtering for quality control, which exclude SNPs with rates of missing values above 5%, rates of heterozygotes above 5%, and allele frequencies smaller than 0.05 or larger than 0.95.

Association mapping

A standard linear mixed model based on the BLUEs of the 261 maize lines estimated across the three

seasons for eleven time points (11, 22, 26, 28, 30, 32, 34, 36, 38 and 42 DAS) independently was used to perform genome-wide association mapping scans (Yu *et al*., 2006). The marker effects were assumed fixed and genotype as random effects. The population structure was corrected using the kinship matrix (Jiang *et al*., 2014). A two-dimensional genome scan based on markers with significant main effects was performed to study marker-marker interactions. The model included the detected main effect QTL as co-factors as well as the main and interaction effects of the marker pair under consideration (Würschum *et al*., 2011). Significance of marker-trait associations was tested based on the Wald F statistic. The Bonferroni-Holm procedure (Holm, 1979) was used to detect markers with significant ($P < 0.05$) main and interaction effects. The detected SNPs were then mapped on maize B73 reference genome (B73 version v1 release 4a.53; http://ftp.maizesequence.org/release-4a.53) and genes in linkage disequilibrium with these SNPs identified.

RESULTS

Complexity of maize genome

This study revealed extensive structural genomic variation dispersed along the maize chromosomes, which includes thousands of CNV/PAV. On average 18,737 CNV/PAV were detected between any randomly selected pair of inbred lines. This study also showed that maize has very complex genome architecture. The detected CNV exhibited genomic architectural complexity in form of smaller CNV within larger ones and CNV with inter-lineage variation in extent of displaying different start and end points (Figure 1). The CNV found in multiple inbred lines displayed inter-lineage variation, with frequently different breakpoints.

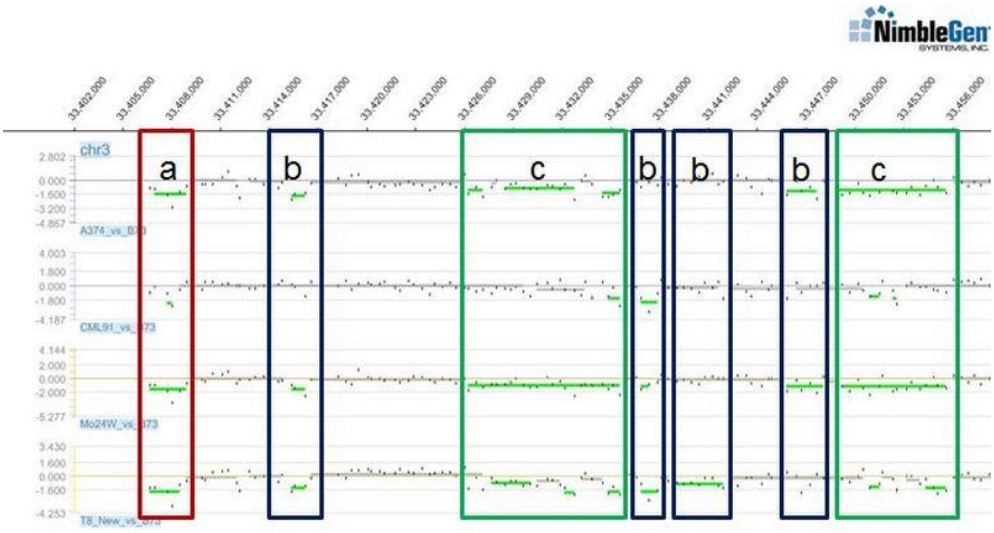

Figure 1: Copy number variation (CNV; 16.6kb) showing different copy number variation (CNV) characteristics in chromosome 3 in four maize inbred maize: (a) CNV displaying CNV in the four genotypes in different copy numbers, (b) CNV indicates that one or more of the inbred lines lack a CNV at this genomic position, and (c) CNV exhibiting a complex genomic architectural CNV in between the inbred lines, probably suggesting different inter-lineage CNV breakpoint or existence of small CNV within larger one. The black dots represent log2 ratio for all probes plotted vs genomic position for all chromosomes per given region for five inbred lines relative to B73 reference genome. The green colour indicates the called CNV. Any CNV call below 0 indicate lower CNV/PAV and any CAN call above 0 means a high CNV for a given inbred line relative to B73 reference genome

Association mapping

Association mapping scan revealed that dynamic QTL for biomass accumulation were well distributed throughout the maize genome, being detected in nine of the ten maize chromosomes. The dynamic QTL analysis showed that different loci with major effects are expressed at different developmental time point (Figure 2). Epistasis mapping scanning also revealed that different loci interact at different developmental time points (Figure 3). The results of the study imply that there is up-regulation and down-regulation of genes controlling complex traits (e.g. biomass accumulation) at different growth and developmental stages of the plant.

A total of 182 genes were found to be harboured in the detected QTL regions, of which 54 have been annotated (Table S1). Two of the genes, *AC215286.2_FG002* and *GRMZM5G859954*, are categorized as cold response genes. The *GRMZM5G859954*, a main effect locus, located at the bottom of chromosome 2, is expressed at early stages of seedling development (11 DAS; Figure 2 and 3). The gene *AC215286.2_FG002* at the top of chromosome 1 interact with other genes at the mid of chromosome 9, at the early stages of seedling development (11 DAS).

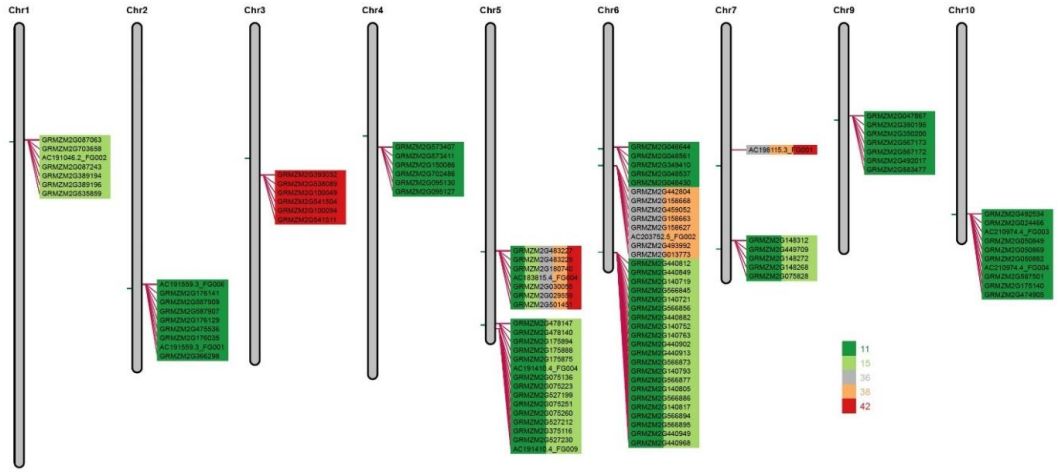

Figure 2: The figure displays significant 12 SNP associations (Holm-Bonferroni =0.05) for maize biomass accumulation and production at different growth time point and genes harbouring the SNP or within 55 kb up- and down-stream of the SNP. The different colours represent 11, 15, 36, 38 and 42 days after sowing as indicate on the key by different colours. The complete gene list is presented in Table S1.

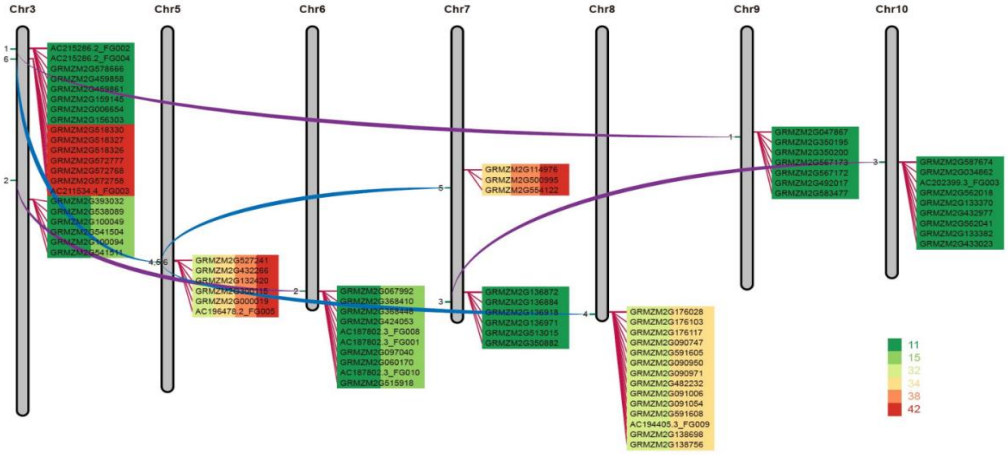

Figure 3: The figure displays 6 pairs of significant interactions (Holm-Bonferroni = 0.05) effects among different loci for maize biomass accumulation and production at different growth time point and genes harbouring the SNP or within 55 kb up- and down-stream of the SNP. Purple and blue connections indicate single and multiple QTL interaction, respectively. The different colours represent 11, 15, 36, 38 and 42 days after sowing as indicate on the key by different colours. The complete gene list is presented in Table S1.

DISCUSSION

This study showed that maize genome is populated with structural variants, CNV/PAV. The findings are consistent with findings from other studies which have shown that plant genomes are rich in structural diversity (Springer *et al*., 2009; Belo' *et al*., 2010; Yu *et al*., 2011). Yet, this type of genetic variants has not been accounted for in genome-wide association

mapping. Though GWAs have been used to dissect many complex traits, majority of these studies uses SNPs-phenotype associations (Bian *et al*., 2013; Busemeyer *et al*., 2013; Liu *et al*., 2014; Würschum *et al*., 2014; Bullucci *et al*., 2015; Bac-Molenaar *et al*., 2015) consequently limiting dissection of trait variation to SNPs genome variation. Understanding the heritability of complex traits will require a more comprehensive assessment of plant genetic variation, including assessment of CNV and dynamic acting genetic factors.

This study demonstrated that even among inbred lines, in which the genetics is simplified to a comparison between two genomes (test inbred line and B73), there is variation in genomic architecture among lines, leading to complex phenotypes. The differences in genomic architecture reflect the complex, often opposing effects of selection, population history, migration and mutation rates. Therefore, these structural variants can account for a large portion of genetic variation among individual genotypes and therefore could account for some of missing heritability. Moreover, such genomic imbalances (CNV/PAV) represent a special class of genetic variants that can potentially affect many genes and pathways in a single individual. This study revealed that CNV can be large, sometimes up to 30 megabases. Consequently, CNV can affect larger genomic sequences and thus have the potential to elicit stronger effects, including changing gene structure and dosage, altering gene regulation and exposing recessive alleles. Several studies have shown that structural variation can alter gene regulation (Henrichsen *et al*., 2009; Zhang *et al*., 2009). In this regard, CNV can be considered as a major source of genetic variation, thus potentially contributing to genetic diversity and consequently contributing to the missing heritability.

The CNV may arise through gene duplication followed by differential loss of duplicated sequence fragments. Gene duplication serves as an evolutionary mechanism for functional innovation (Zhang 2003). Gene turnover in the form of rapid expansion or contraction of gene families has been put forward as a possible explanation of phenotypic divergence (Zhu *et al*., 2007; Perry *et al*., 2008). In human, available data suggest that CNV genes are highly variable among individuals, and enriched genes are associated with environmental interaction (Alkan *et al*., 2009). In human, CNV has been used to explain missing heritability in disorders such as schizophrenia and autism (Stefansson *et al.,* 2008; The International Schizophrenia Consortium, 2008).

A large number of genes acting and interacting at a different plant developmental time were detected in this study for the complex trait, biomass accumulation. This suggested that the expression of a complex trait is a result of action of many genes that may behave differentially during the entire growth and developmental time of a given individual plant, and that gene expression is modified by the interactions genes at different growth time points. Simple phenotypes such as susceptibility to disease is due to genetic variants of large effect (Min-Oo *et al.,* 2003; Diez *et al*., 2003), but complex phenotypes (e.g., variation in lipoproteins) have complex genetic architecture due to the joint action of very many loci of small effect (Valdar *et al*., 2006). The estimation of the positions and effects of QTL is of central importance for marker assisted selection (Zheng and Liu, 2013). In underground networks, most genes work together with close related genes, and it is possible that the effects of one gene on heritability cannot be found without knowing the effects of the others. In complex trait, variation also exists in the extent to which epistasis shapes a phenotype. Epistasis implies one gene can mask the effect of another or several genes can work together. For example, two genes acting at given time may each add one gram to the biomass on their own, but together or even acting at different growth time point they could add five grams. This study demonstrates that the genetic model from final biomass cannot reflect the real gene action during the entire development of the plant. It is, therefore, necessary to understand the dynamics of gene expression for biomass accumulation as a trait at different growth stages as a basis for quantitative trait manipulation.

However, despite the genome sequence information and excellent genomic tools in place for major crop species (Schnable *et al*., 2009; Paterson *et al*., 2009; Ganal *et al*., 2011), genome-wide association studies for dynamically acting genetic factors are constraint by phenotyping. Conventional phenotyping procedures are generally labourious, time consuming, lower throughput, costly, and frequently destructive to plants (e.g., fresh or dry weight determination). Moreover, measurements are often taken at certain times or at particular developmental stages, leading to a phenotyping bottleneck (Furbank and Tester, 2011). This study utilised a high-throughput genotyping platform that is amicable to automation, non-destructive and can generate phenomics data at predetermined intervals (Klukas *et al.,* 2014; Chen *et al.,* 2014; Junker *et al.,* 2015). Using this platform, dynamically acting genetic factors were detected at different plant growth and developmental stages. In addition, cold response genes and a couple of genes

that are involved in transport and photosynthesis were identified. This shows that non-invasive platform is an indispensable tool in studying complex traits, thus supporting the discovery of dynamically controlled genetic factors in GWAs.

CONCLUSION

This study points out two additional possible answers to the missing heritability; i.e. CNV/PAV and dynamic controlled genetic factors. Though genome-wide association mapping approach that can account for these two genetic variants is anticipated to be highly successful in bringing genotype-phenotype existing gap, though, it faces some constraints. Insights into how genetic information in CNV/PAV will be translated into the genetic variability of complex traits are lacking. The genomic architectural complexity implies that in order to be able to detect CNV effects through association testing in larger populations, CNV endpoints need to be precisely delineated to assess information potentially masked by complex CNV architecture, such smaller CNV nested within larger CNV or overlapping CNV regions. Overlapping CNV regions result from inter-lineage variation, which was found to be very frequent in this study.

A promising approach might be to investigate the genetic basis of intermediate phenotypes with lower genetic complexity, such as yield components or metabolites, and link these results back to the complex trait of interest. Population and theoretical genetics approaches may hold the key to finding the missing heritability. However, incorporation of CNV/PAV and dynamically acting genetic factors in GWAS is anticipated to results in better estimation of heritability of complex traits. On the other hand, though automated high-throughput phenotyping platforms are indispensable tool in studying complex traits, the platforms are expensive to establish and thus limited to only a few experiments. Therefore, bridging this apparent genotype–phenotype gap remains a big challenge.

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Supplementary Material

Table S1: Candidate genes residing in the dynamic QTL regions. This include the gene harbouring the SNP or within 55 kb up - or downstream the SNP marker. The panel analysed had a linkage decay of 55 kb.

Gene ID	Gene ID	Gene ID
AC191559.3_FG001	*GRMZM2G483227*	*GRMZM2G566894*
GRMZM2G475536	*GRMZM2G180740*	*GRMZM2G140752*
GRMZM2G366298	*GRMZM2G029559*	*GRMZM2G114976*
GRMZM2G176035	*GRMZM2G483229*	*GRMZM2G500995*
GRMZM2G176129	*GRMZM2G501451*	*GRMZM2G554122*
GRMZM2G587907	*AC183815.4_FG004*	*AC198115.3_FG001*
GRMZM5G859954	*GRMZM2G048537*	*GRMZM2G350882*
GRMZM2G587909	*GRMZM2G048561*	*GRMZM2G513015*
GRMZM2G176141	*GRMZM2G048430*	*GRMZM2G136971*
AC191559.3_FG006	*GRMZM2G048644*	*GRMZM2G136884*
GRMZM2G518327	*GRMZM2G349410*	*GRMZM2G136872*
GRMZM5G813904	*GRMZM2G459052*	*GRMZM2G136918*
GRMZM2G572777	*GRMZM2G158663*	*GRMZM5G884575*
GRMZM2G572758	*GRMZM2G442804*	*GRMZM2G091054*
GRMZM2G518330	*AC203752.5_FG002*	*AC194405.3_FG009*
GRMZM2G518326	*GRMZM2G493992*	*GRMZM2G591605*
AC211534.4_FG003	*GRMZM2G158668*	*GRMZM2G090950*
GRMZM2G572768	*GRMZM2G013773*	*GRMZM2G482232*
GRMZM2G538089	*GRMZM2G158627*	*GRMZM2G138756*
GRMZM2G100049	*GRMZM2G440882*	*GRMZM2G176117*
GRMZM5G895808	*GRMZM2G140721*	*GRMZM2G138698*
GRMZM2G541504	*GRMZM2G566873*	*GRMZM2G090971*
GRMZM2G393032	*GRMZM2G566886*	*GRMZM2G090747*
GRMZM5G835684	*GRMZM2G566856*	*GRMZM2G176028*
GRMZM2G541511	*GRMZM2G440849*	*GRMZM2G176103*
GRMZM2G100094	*GRMZM2G140719*	*GRMZM2G591608*
GRMZM2G573407	*GRMZM2G440812*	*GRMZM2G091006*
GRMZM2G573411	*GRMZM2G440902*	*GRMZM2G047867*
GRMZM2G095127	*GRMZM2G566895*	*GRMZM2G567173*
GRMZM2G150086	*GRMZM2G440968*	*GRMZM2G350195*
GRMZM2G702486	*GRMZM2G140763*	*GRMZM2G583477*
GRMZM2G095130	*GRMZM2G140817*	*GRMZM2G492017*
GRMZM2G527241	*GRMZM5G838319*	*GRMZM2G350200*
GRMZM2G432266	*GRMZM2G566845*	*GRMZM2G567172*
AC196478.2_FG005	*GRMZM2G140805*	*GRMZM2G433023*
GRMZM2G000019	*GRMZM2G566877*	*GRMZM2G133370*
GRMZM2G300115	*GRMZM2G140793*	*GRMZM2G562041*
GRMZM2G132420	*GRMZM2G440913*	*GRMZM2G133382*
GRMZM2G030055	*GRMZM2G440949*	*GRMZM2G432977*
GRMZM2G562018	*GRMZM2G087063*	*GRMZM2G087243*
AC202399.3_FG003	*GRMZM2G389194*	*GRMZM2G703658*
GRMZM2G587674	*GRMZM2G459858*	*GRMZM2G389196*
GRMZM2G034862	*GRMZM2G097040*	*AC191046.2_FG002*
GRMZM2G050882	*AC187802.3_FG010*	*AC191410.4_FG009*
GRMZM2G024466	*GRMZM2G535859*	*GRMZM2G075136*
GRMZM2G492534	*GRMZM5G831408*	*GRMZM2G075260*
GRMZM2G474905	*GRMZM2G578666*	*GRMZM2G527212*
GRMZM2G587501	*GRMZM5G888668*	*GRMZM2G527199*
AC210974.4_FG003	*GRMZM5G858165*	*GRMZM2G175888*
GRMZM2G050849	*AC215286.2_FG002*	*GRMZM2G075251*
GRMZM2G050869	*GRMZM2G006654*	*GRMZM5G842148*
AC210974.4_FG004	*GRMZM2G159145*	*GRMZM2G175894*
GRMZM2G175140	*AC215286.2_FG004*	*AC191410.4_FG004*
GRMZM2G148268	*GRMZM2G060170*	*GRMZM5G889971*
GRMZM2G075828	*GRMZM2G424053*	*GRMZM2G375116*
GRMZM2G148272	*AC187802.3_FG008*	*GRMZM2G478147*
GRMZM2G449709	*GRMZM2G515918*	*GRMZM2G075223*
GRMZM2G148312	*GRMZM2G067992*	*GRMZM2G527230*
GRMZM5G889371	*AC187802.3_FG001*	*GRMZM2G175875*
GRMZM2G156303	*GRMZM2G368410*	*GRMZM2G478140*
GRMZM2G459861	*GRMZM2G368448*	