International Journal of Scientific Research in Knowledge (IJSRK), 1(6), pp. 154-162, 2013
Available online at http://www.ijsrpub.com/ijsrk
ISSN: 2322-4541; ©2013 IJSRPUB
http://dx.doi.org/10.12983/ijsrk-2013-p154-162

Full Length Research Paper

Improving Drought Tolerance in *Sorghum bicolor* L. Moench: Marker-Assisted Transfer of the Stay-Green Quantitative Trait Loci (QTL) from a Characterized Donor Source into a Local Farmer Variety

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Received 19 April 2013; Accepted 11 May 2013

A**bstract.** Drought stress is a major constraint to sorghum production in Kenya, especially during flowering stage. This study aimed at developing drought tolerant sorghum varieties by transferring the stay green trait that confers drought tolerance in sorghum from a mapped and characterized donor source into an adapted farmer preferred variety. The drought tolerance donor source, E36-1 originally from Ethiopia was backcrossed into a Kenyan farmer-preferred variety, Ochuti until BC_2F_1 generation and the stay-green Quantitative Trait Loci (QTL) were transferred through Marker Assisted Breeding (MAB) strategy. Five polymorphic Simple Sequence Repeat (SSR) markers were used to select the 3 stay green QTL of E36-1 found in SBI-01, SBI-07 and SBI-10 linkage groups. In the F_1 generation, two of these QTL, were transferred into three genotypes. In the BC_1F_1 generation, 32 genotypes had at least one QTL incorporated. From a population of 157 BC_2F_1 progenies, 45 genotypes had incorporated either one or two of the stay-green QTL. Despite a few number of genotypes obtained through the backcrosses, the results showed that stay-green QTL and consequently drought tolerance can be transferred successfully into farmer preferred sorghum varieties through MAB.

Key words: Drought; Fore-Ground Selection; Back-Ground Selection; Simple Sequence Repeats; Stay-Green QTL

1. INTRODUCTION

Sorghum is the fifth most important cereal in the world (Dogget, 1988). In Kenya, sorghum is second to maize in production and acreage and is grown mainly in marginal, arid and semi-arid areas where annual rainfall averages 250 mm per season, is erratic and poorly distributed. In these areas, sorghum is an important food and feed security crop (Dogget, 1988). In the recent past, in the Eastern Africa region, a severe drought cycle has been occurring almost every two years affecting sorghum growth at seedling, pre-flowering and post-flowering stages. Sorghum is mostly vulnerable to drought stress at post-flowering stage where reduction of grain yields by more than 50% is likely to be realized (FAO,1999). Nevertheless, sorghum is better adapted to water limiting conditions than other cereal crops because some genotypes have the ability to withstand post-flowering drought stress by retaining high chlorophyll in their leaves and by maintaining the ability to carry out photosynthesis longer. These genotypes aptly, called 'stay-green' also show reduced stalk lodging and are resistant to charcoal rot disease (Rosenow and Clark, 1981). The physiological basis of stay-green is still not clear, though the trait is associated with high cytokinnins and sugar levels. The stay-green trait has recently been characterized in a few genotypes such as B35 and E-36-1 where the Quantitative Trait Loci (QTL) has been mapped (Rosenow and Clark, 1981; Kebede et al., 2001; Haussmann et al., 2002). One of these genotypes namely, E36-1 is a high yielding breeding line assigned to the Guinea-Caudatum race, an Ethiopian sorghum that has been used as a source of stay green (Haussmann et al., 2002) .

QTL mapping of the stay green trait has involved generation of mapping populations such as recombinant inbred lines (RILs), F_2 and backcrosses followed by genotyping (Subudhi et al., 2000). In the fine mapping of stay green QTL in the B35 sorghum variety for instance, a RIL mapping population was developed from the cross, B35 × 7000 and four loci, namely, Stg 1, Stg 2, Stg 3 and Stg 4 , were identified (Xu et al., 2000; Subudhi et al., 2002) . Haussmann et al., (2002) and Kebede et al., (2001) used similar approaches, to map stay green QTL in SC56 and E36-1 sorghum varieties. Once the stay green QTL have been mapped, they can then be targeted for marker assisted selection (MAS) and consequently they provide opportunities to select for drought tolerance in breeding programs (Subudhi et al., 2000).

2. MATERIALS AND METHODS

2.1. Backcrosses generation

Seeds of inbred lines of Ochuti (recurrent parent) and E36-1(donor parent) were sown in a greenhouse at the College of Agriculture and Veterinary Sciences (CAVS), University of Nairobi, in May 2009 and two leaves of each parent were harvested 14 days after sowing. The leaves were preserved in 70% ethanol in eppendorf tubes and transferred to Biosciences east and central Africa (BecA) laboratories at the International Livestock Research Institute (ILRI), Nairobi, for DNA extraction and genotyping. At flowering, artificial hybridization was done by hand emasculation and pollination. Normal bisexual florets of Ochuti were hand emasculated and pollen from E36-1 transferred to the stigma of the emasculated florets. The resulting F_1 seeds were sown and the leaves of 96 F_1 progenies harvested and stored in 70% ethanol in eppendorf tubes for genotyping to confirm the heterozygous F_1 genotypes. At the flowering stage F_1 heterozygous individuals were back-crossed to Ochuti (Recurrent parent) to generate BC_1F_1 genotypes. 128 seeds BC_1F_1 genotypes were sown. 14 days after germination, leaf samples were harvested for genotyping and twenty individuals were selected after foreground and background selections. Upon flowering the twenty selected BC_1F_1 individuals were backcrossed to Ochuti to generate 157 BC_2F_1 individual progenies.

2.2. DNA extraction, PCR and capillary electrophoresis

DNA was isolated from individual plants using the Cetyl-trimethyl Ammonium Bromide (CTAB) mini-prep method as developed by Mace et al. (2004). Determination of the quality and purity of the isolated DNA were done using agarose (0.7%) gel electrophoresis stained with $GelRed_{TM}$ (Biotium). Determination of the quantity was done using the Nanodrop spectrophotometer. All the DNA samples were diluted to a concentration of 10ng/µl.

Table 1 shows a set of 24 SSR markers used for genotyping 96 progenies from Ochuti x E36-1 cross. The markers used were M-13 (-21) tailed (5'CAC GAC GTT GTA AAA CGA C 3') forward primers.

These foreground markers were those that flanked the QTL that confer the stay green trait in variety E36-1.

A 10 µl PCR reaction consisted of: 2 mM $MgCl_2$, 1x PCR buffer, 0.20 µM reverse primer, 0.04 µM forward primer, 0.16 µM florescent dye label, which was either FAM, VIC, PET or NED, 0.04 mM of each of the four dNTPs and 0.2 U DNA polymerase (Sibenzyme®), 30ng template DNA and top up to 10 µl reaction volume, double distilled water was added. GeneAmp® PCR system 9600 (PE-Applied Biosystems) was used for temperature cycling as follows: 15 min at 94°C , 40 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C. The cycling was extended finally for 20 min at 72°C. Following PCR, a few reaction products from each SSR marker were randomly selected to confirm proper amplification and product concentration on a 2% (w/v) agarose gel. Samples that amplified well were subjected to capillary electrophoresis to determine their sizes.

Allele were detected by use of capillary electrophoresis on the ABI 3730 DNA sequencer (Applied Biosystems), which facilitated accurate sizing of the microsatellite alleles to within ±0.3 base pairs. After PCR the amplified products of 3-4 individual primer pairs were co-loaded based on the florescent dye, fragment size and dye florescence strength, to reduce the unit cost of high throughput genotyping. About 1.5 -2.5µl labeled PCR products (depending on the intensity of the bands on the agarose gel) were loaded and mixed also with 7.84µl Hi-Di formamide (PE-Applied Biosystems) that helped to keep the double strands of DNA apart together with 0.16µl GeneScan Liz 500 internal molecular weight size standard (orange) (Applied Biosystems) before denaturing at 94^{o}C for 5 minutes on a GeneAmp® PCR system 9600 (PE-Applied Biosystems).

2.3. Data analysis

Sizing of the PCR products of 35-500 base pairs was done using the GeneScan Liz 500 internal lane size standard with fragment sizes of 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 base pairs. Data analysis was done using GeneMapper® Software version 4.0 where the allele(s) of each genotype in form of peaks were size corrected. The two parental lines, E36-1 and Ochuti were included in each electrophoresis run as controls. Agarose electrophoresis of PCR products showed that the SSR markers for foreground and background selection amplified successfully (Figure 1).

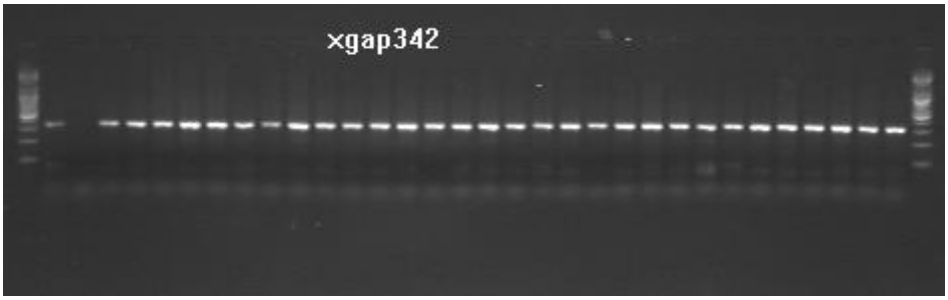

Fig. 1: Agarose (2% w/v) gel showing successful amplification for Xgap342 used in foreground screening.

Fig. 2: Electropherograms from ABI3730 capillary sequencer showing the donor parent allele, recurrent parent allele and the heterozygous progenies

3. RESULTS

The amplicon separated through capillary electrophoresis and analyzed with the GeneMapper® Software identified the polymorphic markers and the heterozygous genotypes at F_1, BC_1F_1 and BC_2F_1generations (Figure 2).

3.1. Foreground selection

Fore-ground selection was done to identify the genotypes that had stay green QTL using the five polymorphic SSR markers that are tightly linked to the QTL.

From ninety six F_1 genotypes that were screened, five genotypes were found to have at least one stay green QTL. The genotypes, named 17, 28 and 32 had two stay green QTL incorporated from linkage groups (chromosomes) SBI-07 and SBI-10 whereas, the genotypes, named 8 and 10 had incorporated only one QTL form linkage group, SBI-10 (Table 2). The true-breeding F_1 progenies were heterozygous for both the recurrent parent (Ochuti) allele and the donor parent (E36-1) allele.

Table 3, shows the number and characteristics of the foreground and background markers used. In the BC_1F_1 generation, out of 128 genotypes screened, 32 were found to have introgressed one or two stay green QTL. Among these, one genotype was found to have introgressed one stay-green QTL from linkage group (chromosome) SBI-07, eleven genotypes had incorporated the SBI-10 QTL whereas another twenty genotypes had incorporated two QTL from SBI-07 and SBI-10 linkage groups (chromosomes).

Table 1: Labeled SSR markers, target alleles and QTL location, for genotyping 96 F1 progenies from Ochuti x E36-1 cross

Marker name	Flourescent dye	Allele sizes		Chromosome with SG QTL
		E36-1	Ochuti	
Xcup24	6-Fam	188	188	SBI-01
Xcup33	Ned	284	284	SBI-01
Xcup032	Vic	155	155	SBI-01
Xtxp043	Pet	152	152	SBI-01
Xtxp088	6-Fam	114	114	SBI-01
Xtxp149	Ned	196	196	SBI-01
Xtxp357	Vic	120	120	SBI-01
Xisep1028	6-Fam	223	223	SBI-01
Xisp0276	Pet			SBI-01
Xisp0324	6-Fam	135	135	SBI-01
Xcup057	Ned	200	200	SBI-07
Xgap342	Pet	304	260	SBI-07
Xisep0328				SBI-07
Xtxp159	Ned	193	195	SBI-07
Xtxp227	Vic	120	120	SBI-07
Xtxp278	Pet	153	153	SBI-07
Xtxp312	6-Fam	235	160	SBI-07
Xgap001	Ned	259	266	SBI-10
Xtxp141	Vic	170	170	SBI-10
Xcup16	6-Fam	252	262	SBI-10
Xisep0639	6-Fam	95	95	SBI-10
Xcup07	Ned			SBI-10
Xcup66	Vic	105	105	SBI-10
Xcup43	Pet	243	243	SBI-10

In the BC_2F_1 generation, a total of 158 progenies were genotyped, out of which 45 progenies were found to have introgressed at least one stay green QTL. Table 4, shows 26 BC_2F_1, of the progenies with these QTL. Nineteen genotypes had double introgression from SBI-07 and SBI-10 chromosomes, whereas 7 genotypes had a single QTL introgression located either on SBI-07 or on SBI-10 linkage groups (chromosomes).

3.2. Background selection

Table 5, shows the BC_2F_1 selected progenies with Ochuti background. This was done to identify progenies that had been advanced from the foreground selection but which also carried the highest proportion of recurrent parent, Ochuti genome. Only 20 genotypes that had over 70% recurrent parent homozygosity were selected for advancement to the next backcross generation.

4. DISCUSSION

Five SSR markers polymorphic in the parental lines and linked to stay-green QTL on linkage groups (SBI-07 and SBI-10) were used to select individuals presumably having the donor allele. These markers selected from the linkage map constructed by Bhattramakki et al., (2000) were able to accurately detect the stay-green QTL at the estimated location as shown in Tables 2 and 4. Foreground selection of the stay-green QTL by flanking polymorphic SSR markers targeted precisely the donor alleles from E 36-1 that were transferred to Ochuti as has been affirmed by Ribaut and Hoisington, 1998. What is of paramount importance in the selection of alleles conferring a quantitative trait is the presence of adequate polymorphism which in itself is critical for conducting marker-assisted selection (MAS) (Young, 1994). As would be expected, from a cross of distantly related parents as that of E36-1 and Ochuti, the progenies should display sufficient heterozygozity at the selected loci. In this study, however, from a total of 96 F_1 genotypes generated, only five were confirmed to be heterozygous for donor and recipient alleles. The markers used here were able to identify true breeding F_1 progenies possessing 50% of each of the parent genome (Frisch et al., 1999).

Table 2: Heterozygous F_1 progenies at Chromosomes SBI-7 and SBI-10

Sample ID No.	SBI-10 QTL				SBI-07 QTL						Introgression
	XCUP16		Xgap001		Xtxp159		Xtxp312		Xgap342		
	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	allele 1	allele 2	
E36-1	252		259		193		235		303		
Ochuti		262		266		195		160		305	
8	252	262	259	266	193	195	235	160			Single introgression SBI-10 QTL
10	252	262	259	266			235	160			Single introgression SBI-10 QTL
17	252	262	259	266	193	195	235	160	303	305	Double introgression SBI- 7 and SBI-10 QTL
28	252	262	259	266	193	195	235	160	303	305	Double introgression SBI- 7 and SBI-10 QTL
32	252	262	259	266	193	195	235	160	303	305	Double introgression SBI- 7 and SBI-10 QTL

Again, in this study, 32 BC_1F_1 and 45 BC_2F_1 genotypes were found to possess the target stay-green QTL. The BC_1F_1 generation had twenty genotypes with double introgression from SBI-07 and SBI-10 linkage groups (chromosomes), one genotype had a single introgression of the QTL from SBI-07 linkage group whereas 11 genotypes had a single introgression of the stay green QTL from SBI-10 linkage group. Single introgressions were those that were heterozygous with regard to a marker linked to one QTL at either SBI-07 or SBI 10 linkage groups whereas double introgression showed heterozygosity for the markers linked at the two QTL in SBI-07 and SBI-10 linkages groups. Twenty BC_1F_1 families were subsequently selected based on the background selection information. Priority was given to the families which had the most of the recurrent parent genome recovered. In the BC_2F_1 generation, there were 19 genotypes that showed double introgression from SBI-07 and SBI-10 linkage groups whereas 10 genotypes had single introgression of stay green QTL located in the SBI-07 linkage group and 16 had a single introgression from SBI-10 linkage group respectively.

Hospital (2005) has argued that the number of individuals genotyped per family is determined by the number of QTL introgressed from the previous family. Some authors (Ribaut and Hoisington, 1998) have also suggested that for each QTL introgression targeted in a back-cross generation, 11 to 22 individuals should be screened to identify the QTL with a 95% ($p \leq 0.05$) confidence. Selecting plants that displayed heterozygozity before pollination reduced the population size by four-fold according to Ribaut and Hoisington (1998). These authors confirmed that if heterozygozity was considered during fore-ground and back-ground selection, the population size was reduced eight-fold for drought tolerance in maize. Frisch et al., (1999) through simulations concluded that marker-assisted selection is, within certain limits, more efficient for large populations than for small populations.

In this study, thirty SSR markers were distributed in the non-QTL regions across the ten linkage groups and were used for background selection in BC_1F_1 and BC_2F_1 generations. This ensured that most, if not all, of the genome of the recurrent parent was recovered by successive backcrosses.

Since, foreground selection done in the F_1, BC_1F_1, BC_2F_1 generations consistently identified genotypes with either one or two possible stay-green QTL, a total of 45 BC_2F_1 progenies were subjected to background screening. The selection of the background was necessary in order to identify lines with introgressed QTL but also having the highest level of the recovered recurrent parent genome in the regions not linked to the stay green QTL. According to the experiments done by Young and Tanksley (1989), selecting the QTL only during at the fore-ground step, would take several generations of backcrossing to eliminate linkage drag.

In this study, the BC_2F_1 plants selected for the stay-green QTL and Ochuti background were selfed and the resultant BC_2F_2 seeds were produced for further evaluation in terms of their stay green phenotype and backcrossing. A visual observation of these progenies in the greenhouse indicated that characters such as the panicle size and plant height of the recurrent parent had been recovered. However, their phenotypic performance can only confirmed after evaluation in the field under replication to assess the expression of the stay-green trait and its effects on terminal drought tolerance.

Table 3: Polymorphic background markers for genotyping BC_1F_1 and BC_2F_1 arising from Ochuti x E36-1 cross

Chromosome	SSR marker	E36-1 allele	Ochuti allele	Flourescent dye
SBI-01	xiabtp378	173	191	Ned
SBI-02	xtxp304	264	213	6-Fam
	xtxp096	152	196	Ned
	Xiabtp168	102	115	Vic
	xiabtp346	190	194	Ned
	xiabtp247	250	238	Ned
	msbCIR238	112	107	Vic
SBI-03	xtxp033	246	249	Vic
	xiabtp29	219	251	Pet
	xiabtp369	269	271	Pet
	xiabtp386	239	249	Vic
	xisep0101	233	231	Pet
SBI-04	xtxp024	169	174	6-Fam
	xtxp021	194	188	Ned
SBI-05	xisp10215	223	220	Pet
	xtxp015	228	238	6-Fam
	xtxp225	195	187	Ned
SBI-06	xtxp176	178	180	6-Fam
	xtxp057	264	266	Ned
	xiabtp424	189	191	Ned
SBI-07	sbAGB02	120	116	Vic
	xiabtp26	115	111	6-Fam
	xiabtp361	289	291	Vic
SBI-08	msbCIR240	126	128	6-Fam
	xiabtp310	230	228	Pet
SBI-09	xtxp258	238	212	6-Fam
	xisep0506	234	227	`
	Xiabtp103	273	248	6-Fam
	xisep0550	204	209	Pet
SBI-10	msbCIR283	132	138	Ned

The efficiency of MAS increases by reducing the genetic distance between the flanking markers used for each target QTL (Hospital, 2005). Practically, the number of markers used should decrease in each successive backcross generation, since once the recurrent parent allele has been fixed at any given non-target region, it is not necessary to continue screening at that locus in the subsequent generations as the locus will remain homozygous regardless of whether this involves selfing or backcrossing to the recurrent parent (Morris et al., 2003). In early generations of this study, the identification of the stay-green QTL required the use of many SSR markers. However, since tightly linked markers were identified in the BC_1F_1, and BC_2F_1 generations, one would expect not only to use fewer markers in successive generations and therefore save on their cost but also to recover of the recurrent genome.

MAS as used here, has the potential to greatly reduce the time required for selecting desirable genotypes with traits of interest (Morris et al., 2003). By conducting fore-ground and back-ground MAS in the early backcross generations, this study identified accurately the stay-green QTL and reduced the undesirable linkage drag. MAS compared with conventional phenotypic selection made it easier to discriminate between individuals heterozygous for one or more of the genomic regions contributing to the stay-green trait.

5. CONCLUSION

In the results reported here, SSR markers assisted in the identification of true breeding F1, BC_1F_1 and BC_2F_1 progenies. Overall, 32 BC_1F_1 and 45 BC_2F_1 genotypes possessed at least one stay-green QTL. Five F_1, 20 BC_1F_1 and 19 BC_2F_1 genotypes incorporated two QTL of the stay-green trait. Twenty BC_2F_1 genotypes that had a high proportion of the Ochuti genome were selected for further advancement. The genotypes that had double introgression of the stay-green QTL are the ones likely to possess drought tolerance. Consequently, if these QTL are stabilized in subsequent generations, these genotypes are also likely to perform better under water limited field conditions.

Table 4: Genotypes introgressing stay green QTL in BC_2F_1

	SBI-10 QTL						SBI-07 QTL				Remarks
Sample Name	**XCUP16**		**Xgap001**		**Xtxp159**		**Xtxp312**		**Xgap342**		
	allele 1	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**allele 2**	**allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	
E36_1	252		259		193		238		303		
Ochuti		262		266		195		163		305	
17_20	251	262	259	266	193	195	238	163	303	305	**D-QTL**
17_20_2	252	262	259	266	193	195	237	163	303	305	**D-QTL**
17_26	251	262	259	266	193	195	238	163	303	305	**D-QTL**
17_26_3	252	262	259	266	193	195	237	163	303	305	**D-QTL**
17_27	251	262	259	266	193		238	163	303	305	**D-QTL**
17_27_1	252	262	259	266	193	195	237	163	303	305	**D-QTL**
17_29	251	262	259	266	193	195	238	163	303	305	**D-QTL**
17_29_6	252	262	259	266	193	195	237	163	303	305	**D-QTL**
17_32	251	262	259	266	193	195	238	163	303	305	**D-QTL**
17_33	251	262	259	266	193		238	163	303		**D-QTL**
17_33_4	252	262	259	266	193	195	237	163	303	305	**D-QTL**
17_36	251	262	259	266	193	195	238	163	303	305	**D-QTL**
28_13	251	262	259	266	193	195	238	163	303	305	**D-QTL**
28_23	251	262	259	266	193	195	238	163	303	305	**D-QTL**
28_29	251	262	259	266	193	195	238	163	303	305	**D-QTL**
28_04	251	262	259	266	193	195	238	163	303	305	**D-QTL**
28_4_1	252	262	259	266	193	195	237	163	303	305	**D-QTL**
32_01	251	262	259	266	193	195	238	163	303	305	**D-QTL**
32_11	251	262	259	266	193	195		163	303	305	**D-QTL**
32_11_1	252	262	259	266	193	195		163	303	305	**S-QTL**
32_15	251	262	259	266	193	195		163	303	305	**S-QTL**
32_19	251	262	259	266	193	195	238	163	303	305	**S-QTL**
32_19_1		262	259	266	193	195	237	163	303	305	**S-QTL**
32_22	251	262	259	266	193	195	238	163	303	305	**S-QTL**
32_26	251	262	259	266	193	195	238	163		305	**S-QTL**
32_26_2	252	262	259	266	193	195	237	163	305		**S-QTL**

Key: D-QTL= double introgression for SBI-07 and SBI-10 QTL; S-QTL= single QTL introgression

Acknowledgements

The authors would like to thank, BIOEARN program of SIDA Sweden, through the Inter-University Council of Eastern Africa (IUCEA) for the award of the grant to conduct the research, the University of Nairobi (UoN) for giving them the time to do this work and Biosciences, eastern and central Africa (BecA) and the International Crops Research institute for the Semi-Arid Tropics (ICRISAT) for technical backstopping.

Table 5: Background screening of BC_2F_1

Sample Name	**No. of Recurrent Parent loci**	**No. of Heterozygous loci**	**No. of Donor Parent (DP) loci**	**% Recurrent Parent (RP) recovered**	**% heterozygous loci**	**%RP+% heterozygous loci**
17_20	25	3	1	83	1	84
17_20_2	27	1	0	90	0.3	90
17_26	12	9	6	40	3	43
17_26_3	22	1	4	73	0.3	74
17_27	9	9	5	30	3.0	33
17_27_1	17	8	3	57	2.7	59
17_29	13	8	3	43	2.7	46
17_32	14	9	5	47	3	50
17_32_3	18	9	1	60	3.0	63
17_32_6	17	8	2	57	2.7	59
17_33	15	6	3	50	2	52
17_33_4	19	5	2	63	1.7	65
17_36	16	8	5	53	2.7	56
28_13	11	10	2	37	3.3	40

28_23	17	12	1	57	4.0	61
28_29	10	12	5	33	4.0	37
28_04	12	8	7	40	2.7	43
28_4_1	22	5	2	73	1.7	75
32_01	15	10	3	50	3.3	53
32_11	12	8	2	40	2.7	43
32_11_1	16	5	5	53	1.7	55
32_15	8	15	3	27	5	32
32_15_1	18	5	2	60	1.7	62
32_15_3	20	2	5	67	0.7	67
32_19	13	11	2	43	3.7	47
32_19_1	19	2	5	63	0.7	64
32_22	10	11	6	33	3.7	37
32_22_1	21	2	3	70	0.7	71
32_22_3	18	9	1	60	3.0	63
32_22_4	22	5	1	73	1.7	75
32_26	11	11	5	37	3.7	40
32_26_2	20	6	1	67	2.0	69
32_07	12	10	6	40	3.3	43
32_7_3	19	4	4	63	1.3	65
32_08	16	7	5	53	2.3	56
32_8_9	17	6	3	57	2	59
32_09	13	10	6	43	3.3	47

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