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A VERSATILE FLUORESCENCE-BASED MULTIPLEXING ASSAY FOR COMBINED GENOTYPING OF SSR AND INSERTION-DELETION POLYMORPHISM (IDP) MARKERS ON CAPILLARY ELECTROPHORESIS SYSTEMS

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Abstract

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During the last decade the number of the IDP markers and their utilization in laboratory practice are constantly growing. Their combination with SSR markers can facilitate saturation of genetic maps. The main disadvantage of both types of markers is the low degree of automation. Although, SSR and IDP markers share similar properties and way of detection, information on multiplexing both types of markers in a single PCR reaction has not been described. Therefore, the present study aimed at increased throughput of the PCR marker assays on a capillary sequencer by simultaneous amplification and fluorescent labeling of SSRs and redundant set of IDP markers in a single closed tube format. Ninety six co-dominant markers, with allele sizes suitable for multiplexing with SSRs, were selected from a public set of 1229 maize IDP markers. Twenty seven of them were polymorphic between the parental lines of a double-haploid population derived from F1 cross of a mutant maize inbred with its progenitor line. Seventeen of the polymorphic IDPs together with nineteen SSRs were used to develop a versatile fluorescence-based multiplexing assay for combined genotyping on capillary electrophoresis systems. Both IDP and SSR markers were successfully co-amplified and fluorescently labeled in 11 multiplex PCR reactions that included up to 4 primer pairs. The robustness of the method was validated by genotyping and map construction in the DH mapping population. The proposed multiplexing method will provide advantages for enrichment of target regions in fine mapping and map-based cloning projects as well as in marker assisted selection. With the increased availability of genome information and genome sequences of multiple varieties, the method could be successfully applied in other model and crop plant species.

Key words: maize, mapping, multiplex PCR, SSR, IDP

Abbreviations: IDP - insertion-deletion polymorphism; MRT™ - multiplex ready technology;
QTL - quantitative trait locus; STS - sequence tagged site

Introduction

The advantages of the PCR based marker such as SSR have made them the most widely used genetic markers (Guichoux, 2011). They have been extensively applied in various kinds of studies including human identification, assessment of genetic diversity and QTL analysis (Gill et al., 1994; Hamblin et al., 2007; Upadyayula et al., 2006). The dramatic expansion of the publicly available genomic sequence data and the progress in the development of bioinformatics tools, during the last decade, allowed development of a new class of STS (sequence tagged site) PCR markers - named insertion-deletion polymorphisms (IDP) or InDel (Bhattramakki et al., 2002). These polymorphisms are caused by insertion-deletion events in a chromosome locus. The allele discrimination is based upon presence/absence variation

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(dominant markers) or size differences of PCR products (co-dominant markers). The size differences range from one to 20 bp (Bhattramakki et al., 2002; Batley et al., 2003), up to several hundred bp (Fu et al., 2006). The IDP markers are more abundant in maize genome compared to SSR markers (Fu et al., 2006). Likewise microsatellites, the IDP markers are highly reproducible and can be reliably scored after electrophoretic separation of the amplified fragments on agarose gels but they have advantage of not producing stutter bands. Because of their large number, more than 38 000 in maize (Setles et al., 2014), IDP polymorphisms are promising marker system, which could provide a high density coverage of the genome (Settles et al., 2014). For these reasons, IDP markers have been developed for number of organisms, including Atlantic salmon, human (Mullaney et al., 2010; Vasemägi et al., 2010), many crop plants like maize, rice, sunflower, citrus, etc. (Bhattramakki et al., 2002; Heesacker et al., 2008; Arai-Kichise et al., 2011; Ollitrault et al., 2012) and have been utilized in both genetic diversity and genetic mapping studies (Fu et al., 2006; Vasemägi et al., 2010; Yamaki et al., 2013; Chen et al., 2014).

Despite the low price of the laboratory equipment needed to score this type of markers, the price of a high-throughput study could be high. This added cost is due to low degree of automation and multiplexing, implying larger staff number and greater expenses for consumables. The multiplex amplification of several loci in a single reaction tube (Edwards and Gibbs, 1994) provides possibility to increase the throughput of PCR assays while reducing the cost for consumables and reagents. In a previous paper (Tsonev et al., 2013), we described a step-by-step protocol for optimization of a cost effective method for multiplexed genotyping of maize mapping populations with SSR markers. Most of the public SSR primer pairs available in Maize GDB database amplify PCR fragments ranging from 100 to 300 bp, thus leaving room for additional markers up to 500 bp when scored on a capillary sequencer. To increase the multiplexing rate of SSR marker assays Wang et al. (2007) redesigned the primer pairs for 60 maize SSR loci allowing the construction of six 10-plex panels for analysis of the genetic diversity on a capillary sequencer. Redundant use of primer sets amplifying SSRs and sequences harboring SNPs was required to construct the multiplex panels for multiplex-ready (MRT™) PCR marker analysis in barley and wheat (Hayden et al., 2008).

The objective of the present study was to increase the throughput of the PCR marker assays on a capillary sequencer by simultaneous amplification and fluorescent labeling of SSRs and a redundant set of IDP markers in a single closed tube format.

Materials and Methods

Plant material and DNA extraction

The maize inbred XM87-136, obtained by chemical mutagenesis and mutation breeding (Tomlekova, 2010; Christov et al., 2014), its progenitor line B37 and their F1 as well as 143 doubled haploid (DH) lines derived from the cross XM87-136 x B37 were employed in the development of the new PCR marker multiplexing method. Collection of plant material and modified CTAB DNA extraction was performed as described previously (Tsonev et al., 2013). Three microliters, containing 30 ng of genomic DNA from each line of the mapping population including the two parental lines, were spotted in 96 well plates, heat dried for 8 min. at 98°C on a PCR cycler with open lid and stored in Ziplock® plastic bags at room temperature until use for PCR reactions.

Selection of IDP markers

Ninety six IDP markers were selected out of 1229 IDP, developed from intronic and UTR sequences of maize genes (Fu et al., 2006). The detailed public information on these markers, including the primer sequences and their polymorphism in a set of 22 diverse inbred lines was downloaded from http://maize-mapping.plantgenomics.iastate.edu. The IDP markers were selected using the following criteria: 1.) Only co-dominant markers were considered; 2.) Fragment size between 300 and 500 bp in B73/Mo17; 3.) Annealing temperature of primers equal or greater than 59°C; 4.) Markers, polymorphic in the set of 22 diverse inbred lines; 5.) Markers in the regions with poor coverage in B37 x XM87-136 mapping population. The published sequences of both forward and reverse locus-specific primers (LSP) were extended at their 5'-ends with additional sequences 5'-ACGACGTTGTAAAA-3' and 5'-CATTAAGTTCCCATTA-3', respectively. The added extensions allow annealing of the generic tag primers used to fluorescently label all PCR products in the multiplex reaction in the second stage of the PCR reaction (Hayden et al., 2008). The tag primers MRT-F 5'-ACGACGTTGTAAAA-3' and MRT-R 5'-CATTAAGTTCCCATTA-3' were also synthesized and the forward tag primer was labeled with one of the following fluorescent dyes FAM, ATTO 565, ATTO 550 or YAKIMA YELLOW (Tsonev et al., 2013). All primers were synthesized by Microsynth, Switzerland.

PCR optimization and capillary electrophoresis

Both uniplex and multiplex PCR reactions were carried out in a volume of 6 µL containing 1x MyTaq™ Reaction Buffer (BIOLINE, UK), 0.15 U MyTaq™ HS DNA Polymerase (BIOLINE, UK) and 75 nmol of each tag primer and 30 ng genomic DNA. In the initial uniplex polymorphism screening

step, 80 nmol of locus specific primers (LSP) for any single IDP locus were used. The PCR was performed with genomic DNA of the parental lines B37, XM87-136 and their F1 on a Verity™ thermal cycler (Applied Biosystems) for a total of 65 cycles with the following profile: 95°C – 3 min, 25 cycles at 92°C – 30 sec, 63°C – 90 s, 72°C – 60 s, followed by 40 cycles at 92°C – 15 s, 54°C – 30 s, 72°C – 60 s and a final extension at 72°C for 10 min. For additional uniplex quality assessment of selected polymorphic markers, the PCR conditions were modified by using lower LSP primer concentration of 20 nmol and the annealing temperature of the first five cycles after the initial denaturation of 50°C, followed by 20 cycles at 63°C. The rest of the PCR program and conditions were identical to the initial screening. The two PCR temperature conditions are designated in the Table 2 as 63°C and 50°C, respectively.

The IDP and SSR markers were combined in five multiplex panels consisting of two or three multiplex PCR reactions each labeled with different fluorescent dye FAM, ATTO 565 or ATTO 550. The multiplex PCR reactions were performed with genomic DNA of the parental lines and F1 and 20 nmol of LSP. For each multiplex PCR reaction, the LSP concentrations were optimized by increase or decrease the concentration of primer pairs for individual loci in subsequent multiplex reactions until similar peak heights for most loci and alleles was reached. After the PCR, reactions belonging to the same panel were pooled and analyzed together on an ABI 3130 capillary sequencer. The post PCR dilution and pooling was done as previously described (Tsonev et al., 2013).

Genetic map construction

The allele information for the markers (17 IDP and 19 SSR) analyzed in the present study was combined with the data from previously analyzed 48 SSRs (overall 84 loci), and a genetic map was constructed, using 143 individuals of a DH population developed from the cross B37 x XM87-136. The linkage map was constructed by Haldane function as implemented in QTL ICIMapping v. 4.0.1.0 software (Li et al., 2007, http://www.isbreeding.net/software/default.aspx). The visualizations and comparisons of the linkage groups were done using Mapchart v. 2.2 software (Voorrips, 2002). The ISU integrated IBM map 2009 of maize chromosome 6 was retrieved from Maizegdb (http://maizegdb.org/cgi-bin/coordinates/map_regions.cgi).

Results

Selection and quality assessment of IDP markers

The effect of application of different filtering criteria for selection of IDP primer pairs that will be suitable for multiplexing with SSR markers is shown on Table 1. After applying constrains on annealing temperature and the size of fragments amplified in the IBM population, 253 primer pairs were selected from the published set of 1229 co-dominant IDPs (Fu et al., 2006),. Out of those, 96 were chosen to amplify loci in the regions with poor coverage in the previously constructed SSR map (Tsonev et al., 2013). The selected 96 IDP loci are listed in Table 2.

Quality assessment of the selected IDP primer pairs was done in two steps. In the first step, the 96 IDP markers were tested for polymorphisms between the parental lines B37, XM87-136 and their F1 cross. Ninety three loci were successfully amplified and labeled in uniplex PCR reactions showing allele sizes in the desired range 250-500 bp (data not shown). Primer pairs for three loci IDP7484, IDP7562 and IDP3939 failed to amplify any PCR product in both parental

Table 1
Summary of the selection procedure and uniplex PCR testing of the IDP primer pairs

Chromosome	Co-dominant	Ta (>59°C)	300-500 bp	PCR tested, Nr	Polymorphic, Nr (%)	Multiplexed, Nr
1	204	177	42	12	2 (16%)	2
2	151	125	32	12	7 (58%)	4
3	145	116	29	14	2 (14%)	1
4	120	97	23	7	3 (42%)	2
5	107	84	17	5	3 (60%)	1
6	122	100	25	6	5 (83%)	4
7	82	68	15	10	0 (0%)	0
8	115	103	36	19	2 (10%)	0
9	99	84	21	7	0 (0%)	0
10	84	68	13	4	3 (75%)	3
Total	1229	1022	253	96	27 (28%)	17

Table 2
List of IDP markers used in the present study sorted by chromosome location

Chromosome	IDP markers
Chr01	IDP3917; IDP3976; IDP1489; IDP3940; IDP6975; IDP8438; IDP4157; IDP241; IDP8939; IDP3798; IDP696; IDP8949
Chr02	IDP3784; IDP3987; IDP3972; IDP2388; IDP3802; IDP8665; IDP3909; IDP8999; IDP3952; IDP3918; IDP7877; IDP7242
Chr03	IDP3958; IDP7717; IDP7814; IDP7645; IDP4912; IDP5018; IDP7873; IDP7166; IDP137; IDP3849; IDP7610; IDP8121; IDP9111; IDP8690
Chr04	IDP6610; IDP7270; IDP4294; IDP1989; IDP1991; IDP3866; IDP342
Chr05	IDP8472; IDP4244; IDP7484; IDP458; IDP8984
Chr06	IDP4179; IDP2428; IDP4196; IDP4247; IDP8945; IDP3956
Chr07	IDP1650; IDP5977; IDP3795; IDP7741; IDP2355; IDP8199; IDP8677; IDP6850; IDP7913; IDP1673
Chr08	IDP7562; IDP3939; IDP6944; IDP4405; IDP4220; IDP4977; IDP2369; IDP6795; IDP9142; IDP8699; IDP8925; IDP6942; IDP6758; IDP7388; IDP8682; IDP8524; IDP8232; IDP3959; IDP1432
Chr09	IDP4038; IDP3862; IDP7298; IDP7283; IDP7595; IDP3957; IDP8021
Chr10	IDP2006; IDP475; IDP2384; IDP3853

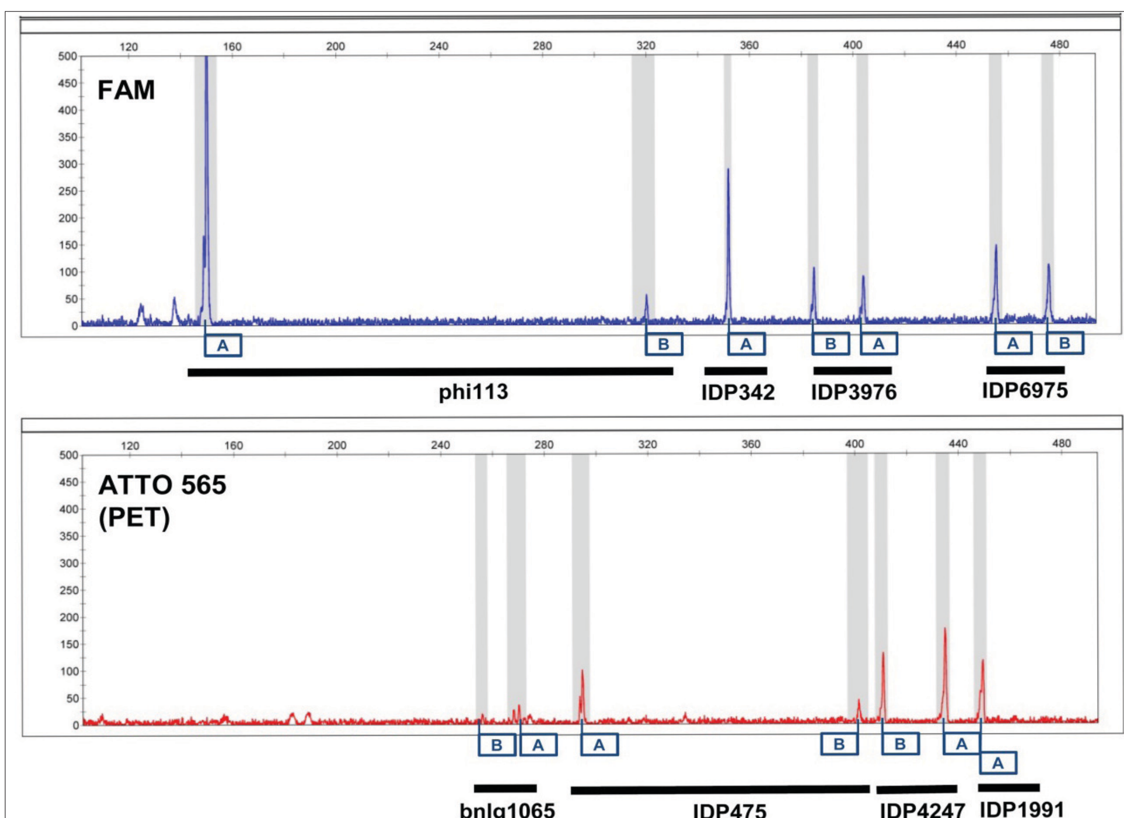

Fig. 1. Example of successful fragment analysis of 2 multiplex PCR reactions including 3 IDP and 1 SSR loci each in the F1 (XM87-136 x B37)

lines as well as the F1 (Table 2). Twenty seven (28%) of the selected IDP produced distinct alleles in both parental lines. However ten of them were excluded from multiplexing for various quality reasons: Three loci (IDP8665, IDP3909 and IDP2428) appeared to be co-dominant, as they produced alleles with different sizes in the parental lines, but only one parental allele was present in F1. Other two that appeared to be dominant (IDP8690 and IDP8682) produced single peak in F1 but no amplification was observed in both parents. The dominant marker (IDP3784) showed no product in F1 while IDP3866 produced multiple background peaks below 300 bp. Although IDP8232, IDP8472 and IDP8984 showed clear co-dominant amplification in the uniplex PCR they failed to amplify alleles with the expected size in several multiplex combinations. The remaining 17 primer sets included 13 co-dominant and four dominant markers (Table 1).

To identify IDP markers that can be multiplexed with SSRs, requiring lower annealing temperature, the amplification of the polymorphic IDPs was additionally tested using alternative PCR conditions including 5 cycles with annealing at 50°C in the first stage of the PCR reaction. In this test, all polymorphic IDP markers with the exception of two (IDP2384 and IDP8472) showed strong amplification of the fragments with the expected size without significant increase of the background and were therefore amenable for multiplexing with SSR primer pairs, having low Ta. The two markers that showed strong unspecific fragments in this condition were still useful in multiplex PCR reactions with annealing temperature of 63°C.

Optimization of multiplexed genotyping assays and genetic map construction

The 17 polymorphic IDP markers were combined with 19 SSR markers in multiplex reactions, according to their allele lengths and annealing temperature requirements. The PCR reactions in each panel (Table 3) were pooled together and analyzed on a capillary sequencer (Figure 1). The initial analysis in multiplex PCR reactions containing 20 nM of LSP for each locus, revealed eleven IDPs and thirteen SSRs that required further optimization of the primer concentration. The concentration of primers was reduced or increased according to the strength of the fluorescent signal. The optimized LSP concentration for each primer pair in the multiplex PCR reactions is given in Table 3.

The DH population was genotyped using five multiplex PCR panels (Table 3). The thirty six markers were amplified in eleven multiplex PCR reactions by combining between 2 and 4 primer pairs (average 3.2) per reaction.

The robustness of the multiplexed fluorescent IDP and SSR methodology was tested for the simultaneous detection and genetic analysis of the 17 IDP markers and 19 SSRs in mapping approach by using a population of 143 DH lines derived from the cross B37 x XM87-136. In all analyzed DH lines, both IDP and SSR markers were clearly identified and accurately assigned to one of the parents. For map construction, the genotype data generated in the present study were combined with the data from 48 SSRs that were previously genotyped. The resulting map had a total length of 1384 cM (Figure 2A). Furthermore, linkage analysis yielded the ex-

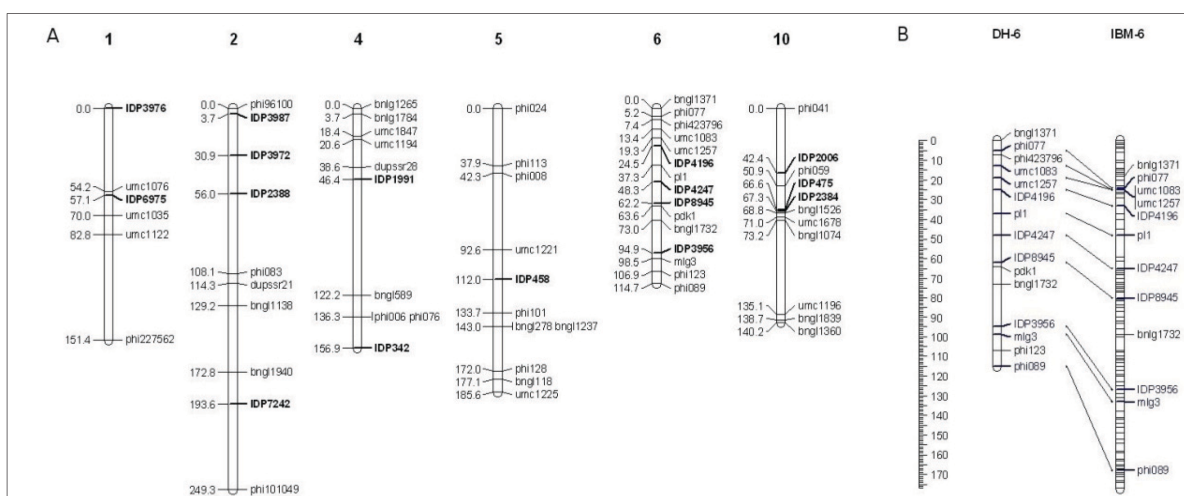

Fig. 2. Linkage mapping of SSR and IDP markers in a DH population derived from a cross XM87-136 x B37.
A) Partial linkage map of maize chromosomes 1, 2, 4, 5, 6 and 10 with IDP markers shown in bold. B) Comparison of marker order on chromosome 6 of the DH population map to ISU Integrated IBM map 2009.

pected results, with all markers mapping as expected when compared to the linkage mapping information of ISU Integrated IBM 2009 maize genetic map (Fu et al. 2006, http://maizegdb.org/cgi-bin/coordinates/map_regions.cgi). Comparison of both maps for maize chromosome 6 is shown on Figure 2B.

Table 3
List of multiplex panels including both SSR and IDP markers

locus	Type	Panel	Dye	Primer pair, Ta °C	Multiplex, Ta °C	LSP, nmol	Allele	
							B37	XM87-136
IDP8945	IDP	G	Red	60	50	60	359/379	333/359
phi008	SSR	G	Red	60	50	10	138	134
phi233376	SSR	G	Red	54	50	20	172	177
bngl1237	SSR	G	Red	54*	50	40	187	230
phi101049	SSR	G	Red	57*	50	40	259	271
phi070	SSR	G	Blue	56	50	40	98	103
umc1083	SSR	G	Blue	62*	50	40	139	141
umc1196	SSR	G	Blue	54	50	10	183	188
phi024	SSR	G	Blue	60	50	10	383	389
IDP2388	IDP	H	Red	60	50	10	384	369
IDP9111	IDP	H	Red	60	50	20	455	412/450
dupssr28	SSR	H	Red	58*	50	20	150	160
umc1076	SSR	H	Red	58*	50	40	281	323
IDP3987	IDP	H	Blue	61	50	20	403	401
IDP3956	IDP	H	Blue	62	50	40	427	423
umc1257	SSR	H	Blue	62*	50	20	191	197
bngl1371	SSR	H	Yellow	54*	50	20	149	155
bngl1191	SSR	H	Yellow	58*	50	40	235	250
IDP458	IDP	I	Red	64	63	20	250/376	-
umc1122	SSR	I	Red	62*	63	10	195	196
phi101	SSR	I	Red	63*	63	10	126	129
IDP3972	IDP	I	Blue	59	63	10	-	377
IDP7242	IDP	I	Blue	60	63	20	336	302
IDP2006	IDP	I	Blue	60	63	40	450	443
IDP1991	IDP	J	Red	61	63	40	449	-
IDP4247	IDP	J	Red	62	63	20	435	411
IDP475	IDP	J	Red	62	63	40	295	401
bngl1065	SSR	J	Red	58*	63	40	267	254
IDP3976	IDP	J	Blue	60	63	20	404	385
IDP6975	IDP	J	Blue	60	63	10	455	476
IDP342	IDP	J	Blue	60	63	10	352	-
phi113	SSR	J	Blue	62*	63	10	150	320
nc009	SSR	K	Red	59*	63	20	175	151
IDP2384	IDP	K	Red	62	63	20	389	435
IDP4196	IDP	K	Blue	63	63	10	405	420
phi227562	SSR	K	Blue	54	63	20	353	341

*No information on optimal annealing temperature available in the databases and publications. Approximate optimal annealing temperature calculated from primer sequences.

Discussion

As a result of the rapid accumulation of sequence data the number of IDP markers in the public databases is constantly growing. In combination with SSR markers they provide abundant resource for further saturation of a genetic map and fine mapping of QTLs for map-based cloning. In the present study we selected a set of public IDP primer pairs for multiplexing with public SSR loci. Selecting primer pairs from a public dataset (Fu et al., 2006) provided two advantages. These IDP loci have already been mapped in the IBM population and were additionally tested for polymorphisms in a set of 22 diverse maize inbreds. Although stringent criteria were applied to select highly polymorphic IDP loci, only 28% of the tested IDP loci were polymorphic between the parental lines of our DH mapping population. Furthermore, a strong chromosome-specific bias was observed in the rate of the IDP polymorphisms (Table 1). The observed polymorphism rate and the chromosome specific bias could be attributed to specificity of the mapping population used in the present study. One of the parental lines of the DH population, XM87-136 was directly developed from B37 by chemical mutagenesis and mutation breeding (Christov et al., 2014). Furthermore, both parental lines were not present in the set of 22 inbreds analyzed by Fu et al. (2006). Moreover, similar polymorphism rate was observed in this DH population for a set of selected 176 SSR loci with high PIC values (data not shown). Absence of amplified fragment in the mutant parent was observed in 3 of the dominant IDPs.

Thus, most probable reasons for those presence-absence variations are mutations in the sequences flanking the InDels. Two of the multiplexed IDP markers generated more than one allele in one (IDP9111 in XM-87-136) or in both of the parents (IDP8945). Fu et al. (2006) suggested that this phenomenon is caused by the presence of paralogous sequences in maize genome. Apparently both alleles of IDP9111 in XM-87-136 are located close to each other, because they did not segregate in the mapping population. Up to 4 markers including both SSR and IDP were multiplexed in a single PCR reaction in the present study and the reliability of allele calling was demonstrated in a genetic mapping approach. This level of multiplexing was comparable to the levels reported in other studies utilizing MRT™ technology for multiplexing SSR loci (Hayden et al., 2008; Tsonev et al., 2013) but lower than that reported for multiplexing only IDP markers in Atlantic salmon (*Salmo salar*) (Vasemägi et al., 2010). The authors achieved multiplexing of up to 12 loci in a single PCR using M13 strategy for fluorescent labeling by selecting InDels ranging between 2 and 11 bp. SSR markers were also used in the same study but were multiplexed separately from the IDPs markers.

The level of multiplexing in the present, proof of principle, study was constrained by the limited choice of non-overlapping SSR and IDP loci. This limitation is mainly caused by the use of published primer sequences that have not been developed for multiplexing. Moreover, since IDP primer pairs have been developed for scoring on agarose gels, the IDPs in the selected range 300-500 bp had relatively large size difference between alleles. The size of insertions-deletions in the loci multiplexed in the present study ranged from 8 to 126 bp with the majority of them being over 20 bp. Moreover, three of the IDP loci had large InDels over 100 bp. The multiplexing rate of both SSRs and IDPs could be dramatically improved by redesigning the primers for shorter (3-10 bp) IDP markers developed by Fu et al. (2006) to include longer flanking sequences and amplify fragments in the range 300-500 bp. Alternatively, *de novo* design of primers for recently published highly polymorphic SSRs (Qu and Liu, 2013) and InDels (Settles et al., 2014) could be used to select markers with high PIC and small size difference between alleles to improve multiplexing capability. With such improvements, multiplexing rates, similar to those reported by Vasemägi et al. (2010) could be achieved for both SSR and IDP markers.

In the present study two marker classes, SSR and IDP, were successfully co-amplified and fluorescently labeled in a single multiplex reaction. The robustness of the method was validated by genotyping and map construction in a bi-parental maize DH mapping population. The proposed multiplexing method will provide advantages for enrichment of target regions in fine mapping and map-based cloning projects as well as in marker assisted selection. With the increased availability of genome information and genome sequences of multiple varieties, the method could be successfully applied in other model and crop plant species.

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