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INSERTIONAL POLYMORPHISMS OF MINIATURE INVERTED-REPEAT TRANSPOSABLE ELEMENTS (*STOWAWAY*- MITEs) IN INTRONS OF SUGAR BEET.

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INSERTIONAL POLYMORPHISMS OF MINIATURE INVERTED- REPEAT TRANSPOSABLE ELEMENTS (*STOWAWAY*- MITEs) IN INTRONS OF SUGAR BEET.

Abstract: Sugar beet has high sucrose content and it accounts for most of the sugar production in the world after sugar cane. European Union is the world's largest sugar beet producer, which accounts for 50% production in the world. A panel of 12 genotypes of *Beta vulgaris* cultivars of sugar beet and fodder beet was used to identify polymorphisms of *Stowaway* miniature inverted-repeat transposable elements (MITEs). In sugar beet DNA is highly polymorphic and highly variant due to its highly repetitive DNA sequences which account for 64% of the genome. Transposable Elements (TEs) are mainly classified into Class I retrotransposons and Class II DNA transposons. MITEs belong to class II, they are non-autonomous TEs. MITEs are the most abundant group of class II elements in the plant genome. *Stowaway* MITEs are derived from and mobilized by elements of the *Tc1/ mariner* superfamily and are one of the significant sources of variation in the sugar beet. MITE copies inserted within introns can be exploited as potential intron length polymorphism (ILP) markers. Polymerase Chain Reaction (PCR) can detect ILPs with primers anchored in exon sequences flanking the target introns. Here, we designed primers for 70 *BvSto* (*Beta vulgaris Stowaway*-like) MITE insertion sites within introns along the sugar beet genome and validated them as candidate ILP markers, to develop a set of markers for genotyping the sugar beet.

Key Words: *Beta vulgaris*, MITEs, Transposable elements, Stowaway mites, DNA, Insertional polymorphisms.

1. Introduction

1.1 Sugar beet

1.1.1 Taxonomy

Sugar beet (*Beta vulgaris* L.) is a biannual crop, which belongs to the family Amaranthaceae, it was developed in Europe in the 18th century from white fodder beets (Oelke et al., 1992). Sugar beet root has high sucrose content, and it is the second source of the world's sugar production after sugarcane (Xiao et al., 2020). Initially, sugar beet was mainly used as a fodder crop but later it was cultivated as a vegetable crop and sugar-producing crop due to its high sucrose content in the root (Oplinger et al., 1991). The chromosome number of Beta vulgaris is 2n=2x=18 (diploid) with a genome size of 758 Mb (Galewski & McGrath, 2020). Sugar beet is an obligate cross-pollinated crop. The breeding of sugar beet is mainly based on mass selection.

1.1.2 Yield

Poland is the third-largest producer of sugar beets in the European Union after France and Germany, with a sugar production of 2.3 Mt per year and 18 operating sugar manufacturers (the European Association of Sugar Statistics CEFS). In the years 2004–2018, sugar beets were cultivated in Poland within an area of about 223,327 ha, but this has fluctuated over time. The multi-annual yield for the period 2004–2018 was on average 54.4 $t \cdot ha^{-1}$, but this ranged from 41.6 $t \cdot ha^{-1}$ in 2004 to 68.3 $t \cdot ha^{-1}$ in 2014 (CEFS; European Association of Sugar Manufacturers), which may be due to the effect of the breeding progress, cultivar adaptation to environmental conditions, or irrigation used in agricultural production. The average annual yield of sugar beet roots in Poland varies by year, e.g., in the 2018 dry growing season, the average yield of sugar beet amounted to 59.9 $t \cdot ha^{-1}$, which constituted 88.2% of the yield in 2017 (Zarski et al., 2020).

1.1.3. Land preparation

Sugar beet can be grown in various soils ranging from sandy loam to clay. Loam soil rich in humus is the ideal soil for the growth of Beta vulgaris (M. Li et al., 2020). Sugar beet grows best on soils with a pH of 6.0 to 8.0, but it does not grow well on highly acidic soils (Oelke et al., 1992).

1.1.4. Sowing and seed rate

Sugar beet is grown from seed, and the seedbed is prepared from deep ploughing. Seeds must be disinfected before sowing to avoid black root disease. Seeding rates vary from 0.4 to 0.9 kgs of seeds per acre.

1.1.5. Environmental conditions

Sugar beets can be grown in a wide range of climatic conditions. For, sugar beet growth can be stopped due to hard freezing. From the day of planting, it takes 70 to 90 days for total growth.

The optimal daytime temperature for the growth and maximum yield of sugar beet is 18 to 26°C with bright sunny days. And the best night-time temperature should be 5 to 10°C (Oplinger et al., 1991).

1.1.6. Water requirement

Sugar beet is a plant with increased water requirements due to its long growing season and high yield potential. Making full use of its yield potential as well as of the applied agro-technical treatments including nitrogen fertilization depends on many factors, e.g. genetic diversity of hybrids (Tarkalson et al., 2014) and the adaptation abilities of cultivars to an individual environment (Studnicki et al., 2019), but it most heavily depends on the type of soil or location and above all, on the availability of water during the period of the high demand of the plants. This water availability to plants is subject to the rainfall volume and distribution during a growing season, or in the case of rainfall shortages, it relies on the use of irrigation if possible. In Poland, under conditions of a moderate climate, the average rainfall totals in the growing season are in the range of 350–400 mm; however, they are distinguished by great temporal and spatial variability. Therefore, irrigation of sugar beet has a supplementary and intervention nature and is applied only when dry periods occur in the growing season (Zarski et al., 2020). The requirements, results, and perspectives of plant irrigation in the areas are characterized by distinct water deficits. The amount of water in the sugar beet root zone in irrigated plots held throughout plant growth is between 0–40 mm (Zarski et al., 2020).

1.1.7. Nutrient requirement

Good nitrogen management is most important in sugar beet production to bring optimal yield (Hergert, 2010). Nitrogen shows a direct relationship with sugar beet yield because it is the nutrient most limiting plant productivity (Connor et al., 2011). Too little application of nitrogen results in a reduction of root tonnage and the application of more nitrogen results in a reduction of sugar concentration (Hergert, 2010). Crops must be maintained within 4 to 6 weeks before harvest without excess N (Ulrich, 1955). While harvesting time approaches, N availability should decrease to enhance sucrose formation (Ulrich, 1955). Nitrogen recommendations should be based on the soil type, variety, and yield potential (Westfall & Building, 2015). Phosphorus and potassium are major nutrients required for sugar beet production. In the early years, soils were low in phosphorous. Animal manures generally contain a significant amount of phosphorous, but it supplies a limited amount of N and P. so commercial fertilizers of phosphorous usage led to high soil P (Hergert, 2010). High rainfall regions having >500 mm per year have lost basic cations during the soil development process. So these soils require liming and K (Hergert, 2010). NPK requirement is shown in table 1.

Table1. NPK requirement for sugar beet growth

Sugar beet yield goal ton/acre	Soil N plus fertilizer N needed* Kgs/acre/2 ft	Phosphorus P_2O_5 Kgs/acre	Potassium K_2O Kgs/acre
16	43	15	38
17	45	15	40
18	49	18	43
19	52	18	45
20	54	20	47
22	58	22	52

1.1.8. Disease and pests and their management

Sugar beet can be attacked by many diseases and insect pests. Some serious diseases which reduce the yield of sugar beet are black root rot, which is caused by a fungus having characteristics of lesions in the stem near the soil. Another disease is Cercospora leaf spot, which is also a fungal disease in which the leaves become greenish yellow and root weight

and sugar content are reduced, are most serious, and can cause great damage if not controlled. The cause of Cercospora depends on the presence of susceptible cultivars, adequate inoculum, and environmental conditions characterized by periods of high humidity or leaf wetness periods longer than eleven hours and warm temperatures greater than 60°F. To control Cercospora disease crop rotation can reduce the overwintering of inoculum; at least three years of rotation is needed to reduce the quantities of infested residue. Also using leaf spot-tolerant varieties can reduce the infestation (Rangel et al., 2020). Another serious disease is Rhizomania. It is a soil-borne disease that causes severe yield losses in the absence of effective control measures. It is caused by beet necrotic yellow vein virus (BNYVV) (Scholten & Lange, 2000), which is transmitted by the obligate root-infecting parasite *Polymyxa betae*. Symptoms of Rhizomania are bearded root, internal vascular discoloration, tumorous outgrowths, and constricted shape. The control of Rhizomania is now achieved almost exclusively through the use of resistant cultivars. A single dominant resistance gene, *Rz1*, has been used to manage the disease. Some common pests which attack the sugar beet crop are cutworms, root maggots, flea beetles, wireworms, root aphids, white grubs, and beet webworms. Precautions must also be taken against damage by worms, beetles, and nematodes (Youssef et al., 2020).

1.2 Transposable elements

Insertional polymorphism is a type of genetic variation in which a specific nucleotide sequence is inserted from one part of the genome to another part of the genome. Miniature inverted-repeat transposable elements (MITEs) are found in the introns of many genes. All these elements, earlier referred to as junk DNA as a part of introns are removed by RNA splicing so that they are not present in the mature mRNA. Transposable elements (TEs) were first discovered by Barbara McClintock maize (*Zea mays*; McClintock, 1951). TEs are also called mobile DNA capable of changing positions within the host genome through continuous integration (Grzebelus, 2018). There are two classes (class I and class II) of TEs based on their transposition mechanism (Zhao et al., 2016).

1.2.1 Class I Transposable elements:

Class I TEs transpose by an RNA intermediate: as a result, a new copy is integrated into a new site while the original sequence remains intact at the donor site. Instead of copying the DNA using replication, they produce RNA by transcription. Once they make RNA, RNA is used to make a complementary DNA copy (cDNA) using a reverse transcriptase enzyme. A double-stranded DNA sequences are subsequently produced which will insert in a new position in the genome. That is why it is called a 'copy and paste' transposition. Class I is divided into five orders. They are long terminal repeat elements (LTR), long interspersed nuclear elements (LINE), short interspersed nuclear elements (SINE), Dictyostelium intermediate repeat sequence (DIRS), Penelope-like elements (PLE)(Kumar & Bennetzen, 1999). In plants, superfamilies like *Ty1-Copia* and *Ty3-gypsy* of LTR retrotransposons are more successful and constitute the major fraction of all plant transposable elements (Zhang et al., 2017). LINEs and SINEs are repetitive sequences that act as markers to find out differences in different individuals in a population. The importance of LINEs and SINEs is they jump from one place to another, which gives variations (Feschotte et al., 2003).

1.2.2 Class II Transposable elements:

Class II TEs are called as DNA transposons (Feschotte et al., 2002). DNA transposons transpose by the 'cut and paste' mechanism: because they move from the donor site and reintegrate at the acceptor site. These elements are less abundant when compared with LTR retrotransposons. Class II is divided into two orders terminal inverted repeat elements (TIR) and Helitrons. TIRs are further divided into five superfamilies called *hAT, Mutator, CACTA, PIF/Harbinger*, and *Tc1- mariner*. The two largest MITE families are *Stowaway* and *Tourist*. *Stowaway* family is identified as a member of the *Tc1/Mariner* superfamily, and *Tourist* is identified as a member of the *Harbinger* superfamily (Grzebelus, 2018).

Each group of TEs contains autonomous and non-autonomous elements. Autonomous elements have ORFs (open reading frames) that encode the products required for transposition (Wessler, 2006). Autonomous transposons can transpose without the help of

any other gene. Whereas, non-autonomous elements do not encode transposition protein, but they can transpose because of the cis-sequences that are necessary for transposition (Wessler, 2006). Non-autonomous transposons require the help of other autonomous elements to transpose.

1.3 *Stowaway* MITEs:

In a maize mutant, the insertion of 128 bp in a waxy gene called *wxB2* allele led to the identification of *Tourist* elements in the intron regions of the genome of this grass species. A 257 bp insertion in the sorghum led to the discovery of *Stowaway* in the genes of flowering plants (Feschotte et al., 2007). The two largest MITE families Tourists and *Stowaways* were identified as the members of the *PIF/Harbinger* and *Tc1/Mariner* super families respectively (Jiang et al., 2004). *Stowaway* MITEs were first described in the maize genome (Bureau & Wessler, 1994) as elements shorter than 500 base pairs long. *Stowaway* MITEs are present in thousands of copies per genome. A total of 22,000 *Stowaway* MITEs are classified into 34 families in the *Oryza sativa* genome (Feschotte et al., 2003), and 18,000 *Stowaway* MITEs are classified into 18 families in the *Triticum* species genomes (Feschotte et al., 2003)

Stowaway elements are small in size (<600bp). The terminal sequences for *Stowaway* elements are "CTCCCTCC…GGAGGGAG". When the non-autonomous *Stowaway* elements were initially identified from plants, it was very unclear which types of autonomous elements were associated with a *Stowaway*. Therefore, they were classified as MITEs based on their structural characteristics. Later it was revealed as *Stowaway* elements are related to *Tc1/Mariner*-like elements. In many plant genomes, even though they account for a limited fraction due to their small size, they are present very abundantly. *Stowaway* elements are derivatives of autonomous *Mariner* transposable elements based on computer system analysis, revealing sequence homologies restricted to TIRs and 5'-TA-3' target sites of both autonomous and non-autonomous plant elements (Turcotte et al., 2001).

There are three previously described families of MITEs in *Beta vulgaris* called as *VulMITEs I, VulMITEs II*, and *VulMITEs III* derived from the *Vulmar* family of *mariner* transposons. *VulMITEs I* are typical *Stowaway*-like MITEs, *VulMITEs II*, and *VulMITEs III* are rearranged *Stowaway* elements of increased size (Menzel et al., 2006). The *VulMITEs I* group consists of 19 clones ranging from 237 base pairs to 307 base pairs. All clones have AT content of

66.6% and 70%. *VulMITEs II*-1 and *VulMITEs II*-2 are 1008 base pairs and 1011 base pairs in length sharing 95.8% similarity. *VulMITEs III* group consists of two members with 1124 base pairs and 1167 base pairs lengths with a similarity of 63.1% (Menzel et al., 2006). These entire *Stowaway* MITEs were distributed all over the *Beta vulgaris* genome as observed by performing fluorescent in situ hybridization (FISH; (Schmidt et al., 1994). The transposition of *Stowaway* MITEs into a dispersed repeat was identified by comparison of *Beta vulgaris* different cultivars and the mobilization of the MITEs within the DNA during the domestication of sugar beet. *Stowaway* MITE-mediated amplification of repetitive DNA has a significant impact on genome size and evolution (Menzel et al., 2006).

This study shows how the abundance of class II TEs may serve as a tool for the relatively rapid and low-cost development of gene-derived molecular markers, for effective use in sugar beet genotyping studies. Insertions within introns may provide significant polymorphisms. Intron-length polymorphism (ILPs) can be exploited as genetic markers used for gene mapping (Wydner et al., 1994) and population genetic surveys (Lessa, 1992). ILPs take advantage of the different rates of evolution of exons and introns that can result in conserved exon nucleotide sequences adjoined to more variable intron sequences. ILPs can be detected by the polymerase chain reaction (PCR) with a pair of primers anchored in the exons flanking the intron of interest (Wang et al., 2005). ILP markers are unique due to their gene-specificity, codominance, convenience, reliability, and cost-efficiency. Furthermore, ILPs are characterized by high transferability among related plant species (Yang et al., 2007; Gupta et al., 2011).

ILP markers are used to check the polymorphisms in sugar beet cultivars. ILP markers are codominant in nature. Intron length polymorphism (ILP) is growing because it's not only showing similar advantages to SSR but also shows very unique qualities like high plant interspecies transferability. ILP markers are also fast, reproducible, convenient and ready to use.

1.4 Double haploids in Sugar beet:

Double haploids are formed when haploid cells undergo chromosome doubling. In sugar beet, the F1 hybrids are developed by crossing two homozygous parental lines. To develop homozygous lines it takes a minimum of 8-12 years with conventional methods. So, the double haploid method offers a time-saving approach to produce homozygous lines.

2. Aim

The aim of this work is to develop a genotyping system utilizing *Stowaway* insertional polymorphisms in introns of sugar beet. Genotyping can be used to find polymorphisms, which are useful to confirm homozygosity (e.g. during the production of double haploids).

3. Material and methodology:

3.1 Plant material

Sugar beet was grown in a growth chamber from seeds. Twelve genotypes of sugar beet (*Beta vulgaris* L.) of diverse origin with different phenotypic traits were used for this study. The names and their resistance and the origins of the samples are shown in table 2.

Table 2. The name and the origin of the sugar beet accession

Nr	Name	seed	Description	Resistance	Origin
1	SB1	multigerm	DH	-	KHBC
2	SB2	multigerm	F3	-	KHBC
3	SB3	multigerm	F3	-	KHBC
4	SB4	multigerm	F4	*Rz1*	KHBC
5	SB5	multigerm	F4	*Rz1*	KHBC
6	SB6	multigerm	F5	*Rz2*	KHBC
7	SB7	monogerm	F5	*Cercospora*	KHBC
8	SB8	monogerm	F5	*Cercospora*	KHBC
9	SB9	monogerm	F5	*Cercospora*	KHBC
10	SB10	monogerm	F4	*Cercospora*	KHBC
11	FB1				MHR
12	FB2				MHR

3.2 DNA Extraction:

Genomic DNA was extracted from seedlings using the CTAB protocol (Torres et al., 1993). DNA quality was checked by using gel electrophoresis. From each genotype of sugar beet 120 mg of DNA were extracted.

CTAB buffer preparation (CTAB buffer) -100ml:

CTAB (Cetyl trimethylammonium bromide)-2g

1M Tris HCl pH 8 – 10ml

5M NaCl – 28ml

0.5M EDTA -4ml

To dissolve CTAB powder, it is necessary to stir and warm up the solution.

DNA extraction of Sugar beet:

1. 10 Sugar beet and 2 fodder beet samples were collected for DNA extraction.
2. 200mg of each plant sample was taken and ground with liquid nitrogen.
3. Then transferred those grounded samples into an Eppendorf tube and added 700 µl of CTAB buffer.
4. The mixture was vortex and incubated at 65°C for 20 min in a water bath.
5. The samples were then centrifuged the samples at 10000 rpm for 10 min.
6. The supernatant was collected and transferred to another tube and added an equal amount of chloroform and isoamyl alcohol was added.
7. The solution was vortexed and centrifuged at 10,000 rpm for 10 min.
8. After centrifuge the solution was divided into an upper aqueous layer and a bottom organic layer.
9. The upper aqueous layer was transferred into another tube without disturbing the lower organic layer.
10. Then 600 µl of cold ethanol (-20℃) and 150 µl of NaCl was added to the aqueous layer.
11. This solution was mixed and centrifuged at 13,000 rpm for 10 min.
12. DNA pellets were observed at the bottom of the tube after centrifugation.
13. The supernatant was discarded and washed the DNA pellets with 600 µl of 70% ethanol and centrifuged at 10,000 rpm for 5 min.
14. After the centrifugation ethanol was discarded, the pellet was dried and it was added with 50 µl of 1X TE buffer.
15. The samples were then stored at 4°C.

3.3 Primers development

Primers for performing polymerase chain reaction (PCR) were developed using the National Centre for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) by inserting chromosome names, open sequence, and by using beginning and ending positions of Sugar beet DNA sequence. By using the NCBI website almost 70 pairs of primers were designed for the studies. The primers are listed below.

PRIMER NAME	SEQUENCE
Bv_chr2_Sto4_1_1_F	TGCTGATAAAATTGGTGGTGATGT
Bv_chr2_Sto4_1_1_R	GCCTTTGCCAGTTTTTCGGG
Bv_chr2_Sto4_1_2_F	CGGCTTTGTCATATTCTCGCT
Bv_chr2_Sto4_1_2_R	TTCGACAGAGAAACTCGCCT
Bv_chr2_Sto4_1_3_F	CATAACCTGTCCCCCATGCAA
Bv_chr2_Sto4_1_3_R	ACTGGAGTGGTAATCCACAGG
Bv_chr3_Sto4_1_4_F	GCTGACTAAGAAGGCCCCAA
Bv_chr3_Sto4_1_4_R	TGCCGCCGTTATCTTAATGC
Bv_chr3_Sto4_1_5_F	TGACCGTGTTAGTGATCGCC
Bv_chr3_Sto4_1_5_R	GTGAGCACAGCTTGATTCTCC
Bv_chr4_Sto4_1_6_F	AGTGTTCCTCAAGGCGATGC
Bv_chr4_Sto4_1_6_R	TCCACAACAATCACCTTCCCA
Bv_chr4_Sto4_1_7_F	GCTCCCACATGTAACCAGTTCT
Bv_chr4_Sto4_1_7_R	AGCTTGCATTTTGTGCACTCTT
Bv_chr4_Sto36_1_8_F	ATTGAGGCAACAAAGGTGGGT
Bv_chr4_Sto36_1_8_R	TGACAGTTGCTCTTATGGCAGA
Bv_chr5_Sto4_1_9_F	CGCCTCTGTGAATGGATGCTT
Bv_chr5_Sto4_1_9_R	TATCGATGGGGCTTCTTTTGCT
Bv_chr5_Sto10_1_10_F	ATGATTTCATTTTTGGGGCTGCT
Bv_chr5_Sto10_1_10_R	CTAGGACCTCTACCATCCTCTTTC
Bv_chr1_Sto3_2_14_F	TTTCCCATCCCCCAATATCCAG
Bv_chr1_Sto3_2_14_R	CTTTGAGTTGAAGGTGGAAGCG
Bv_chr2_Sto3_2_29_F	GGAATATGCCGCTTTCCTCCT
Bv_chr2_Sto3_2_29_R	TCAAGTAGCCGAGGCATCTC
Bv_chr2_Sto3_2_32_F	TGCACATTTTCCCATTATCCAGC
Bv_chr2_Sto3_2_32_R	AGTTTAAAGAGTGCTGCTCCTGA
Bv_chr3_Sto3_2_45_F	AGGATTGTTCTCAAGCCCAAC
Bv_chr3_Sto3_2_45_R	TGGTCGTGTAGACATGAAAGCC
Bv_chr4_Sto3_2_62_F	GGAAGTGGCAAGATGCTGAAG
Bv_chr4_Sto3_2_62_R	GCTGTAAACCCTTCATTGGTCA
Bv_chr4_Sto3_2_64_F	ACTTGTGGTCCTAGTCATGGA
Bv_chr4_Sto3_2_64_R	ACGCAGGCTTTGGAATTATGG
Bv_chr4_Sto3_2_65_F	CCTTGCCATGGCTATTTGGTTT
Bv_chr4_Sto3_2_65_R	GCACTGGATCGTAGCCATGAG
Bv_chr4_Sto3_2_69_F	TGCCTACTAGGTAATAAGCTCCCAG

Bv_chr4_Sto3_2_69_R	ATTGATGGCATTCAAGCTGTATTGT
Bv_chr4_Sto3_2_78_F	TCCTTGTTGTCTATTGTAATTGGCT
Bv_chr4_Sto3_2_78_R	TCAAGGAAAATCTACTCCACGCT
Bv_chr5_Sto3_2_89_F	TCACTTTGCTGGACTGAAGGC
Bv_chr5_Sto3_2_89_R	AATTTTCTCTGGCTGGCCG
Bv_chr5_Sto3_2_90_F	GAAGACGGAAACGCCTACTTTG
Bv_chr5_Sto3_2_90_R	ACGATTCCCATGATCAGGCTT
Bv_chr5_Sto3_2_99_F	AGGGTCGAGATTACTGGTCCT
Bv_chr5_Sto3_2_99_R	CCCAGCTTGGTGATAGTGCAT
Bv_chr5_Sto3_2_109_F	ATTGCTGCTGGGATTTCTATGC
Bv_chr5_Sto3_2_109_R	TGAGATGCCTTCGACCAAAGT
Bv_chr5_Sto3_2_112_F	TGCACTTGATGTCCTCTGGC
Bv_chr5_Sto3_2_112_R	GTGTTTTCCCCACCCAGTCA
Bv_chr6_Sto3_2_118_F	CCTTCCTATGCAGGAACCTTAC
Bv_chr6_Sto3_2_118_R	CACCTTATGATTGCCAGAGCC
Bv_chr6_Sto3_2_123_F	TGATCATCACCGCCGCTTTA
Bv_chr6_Sto3_2_123_R	CCCCATACCACTGTGTATAGCC
Bv_chr6_Sto3_2_124_F	GTCGGAGACATTTACGGTGGT
Bv_chr6_Sto3_2_124_R	ATAAGCCGCAATAGGGATCGG
Bv_chr6_Sto3_2_130_F	GGAATCCTGCTTTCTTCGGC
Bv_chr6_Sto3_2_130_R	GCGATCGAGTTAGCACTTCCA
Bv_chr7_Sto3_2_144_F	ACCCGGATAGATCTCTGTGTAGA
Bv_chr7_Sto3_2_144_R	TGGGAGTCTCTCTAACGCAT
Bv_chr7_Sto3_2_151_F	AAAGAACTGAGGCCACTTGGA
Bv_chr7_Sto3_2_151_R	TCATTGAAGGCATCAAGGGAT
Bv_chr8_Sto3_2_157_F	CGTGTGTGAGAACCATCCAGAA
Bv_chr8_Sto3_2_157_R	AGGGCTGTCTGTAATTGAGACTT
Bv_chr8_Sto3_2_165_F	GCCACTGGTTATGATGGGAAG
Bv_chr8_Sto3_2_165_R	GCTAGCCACTTACACCGCAG
Bv_chr8_Sto3_2_171_F	TCCTGAGTTCCACTTGGTGC
Bv_chr8_Sto3_2_171_R	CAGTTGTTAGAAAGTACATCGCCA
Bv_chr9_Sto3_2_181_F	ATTGCCATTTGGGTCACTGC
Bv_chr9_Sto3_2_181_R	TTAAGGAGAAGCTTATGCGCC
Bv_chr9_Sto3_2_186_F	GGCTCTTCCTGCTATAGCCTTT
Bv_chr9_Sto3_2_186_R	AGTTTGCACTGAACTCAGGTTTC
Bv_chr9_Sto3_2_192_F	TTGCTTTGTTGAGTAAGTGGGC
Bv_chr9_Sto3_2_192_R	GGGGGAATAAGGTTTCTTCACAAG
Bv_chr1_Sto36_2_17_F	ACCAAAAACGCTCTCAGCAA
Bv_chr1_Sto36_2_17_R	ATTGCCTCTGATCCTGCACC
Bv_chr1_Sto36_2_21_F	TTGCGGAGTGGCTAGTTTCG
Bv_chr1_Sto36_2_21_R	TGTGCTGCCCAAGCAAAATATC
Bv_chr2_Sto36_2_23_F	GGGTTTCCATAGACTTGATCCGTA
Bv_chr2_Sto36_2_23_R	GCTCGTCTTATCGAGACCCA
Bv_chr2_Sto36_2_30_F	GCCTCTTCACCTTTAGGCACT
Bv_chr2_Sto36_2_30_R	GGTTTGCGTGAATGGGATCG
Bv_chr4_Sto36_2_70_F	AGTTGAGCACCCCCAATGAG

Bv_chr4_Sto36_2_70_R	GCTCGAGAACTTCTACCAAAACC
Bv_chr4_Sto36_2_74_F	TGTTGACGAGATTTTGTTGGCA
Bv_chr4_Sto36_2_74_R	CCTGATGGGTACACTTGGAG
Bv_chr4_Sto36_2_76_F	GGTAGGTTGATGAGCTCAGGT
Bv_chr4_Sto36_2_76_R	ATATTGCGCTCAAGCAGAGGA
Bv_chr5_Sto36_2_84_F	AGCTACGAGAGTAACCTTCGG
Bv_chr5_Sto36_2_84_R	TTGCCTGGCGTCATACTGAA
Bv_chr5_Sto36_2_88_F	TGACAGCTGACGGTGTTCC
Bv_chr5_Sto36_2_88_R	GAAGTGTTGCTCAAAATGCGG
Bv_chr5_Sto36_2_94_F	ATTACTTTTTGCATGTTGGAGCTG
Bv_chr5_Sto36_2_94_R	CAGCACGTCAACACCGAGAA
Bv_chr5_Sto36_2_98_F	TGGATCCATTAACCCGTGGC
Bv_chr5_Sto36_2_98_R	CCCGACGACGTTTCCTTCAT
Bv_chr5_Sto36_2_100_F	TGTGATTGATAGATACTGCGTCTT
Bv_chr5_Sto36_2_100_R	AATATTTGTTCCCAGTGGATGGT
Bv_chr7_Sto36_2_132_F	TCTCCAGTTGTGGTCTTTGGAG
Bv_chr7_Sto36_2_132_R	GAGCCTAAACCAAGCCGCTAA
Bv_chr7_Sto36_2_134_F	CCTTTGAATTCGGTGCCGGG
Bv_chr7_Sto36_2_134_R	AGAAGGTTACAATCCTGCCACA
Bv_chr7_Sto36_2_138_F	TCCATTCGAAGCAGTTCTGAGT
Bv_chr7_Sto36_2_138_R	GCTGGAGTAAGAAAGAGATGCCT
Bv_chr8_Sto36_2_154_F	GAAGCCCAATTTCGAAGGACG
Bv_chr8_Sto36_2_154_R	ACTCAGGCCTTCAGTACAACTC
Bv_chr8_Sto36_2_162_F	CACTGTCTGTGTGTGGCATC
Bv_chr8_Sto36_2_162_R	TCCCTTGAACGACGTTTGCG
Bv_chr9_Sto36_2_175_F	TCCCTCTAAAAGTTCAGAGTCTTC
Bv_chr9_Sto36_2_175_R	TTTGCAGAAAGACCACTGCC
Bv_chr9_Sto36_2_190_F	TAAAGTGCCAAGGCATGACCA
Bv_chr9_Sto36_2_190_R	AAGTCTCATGAAGGGTTGGAC
Bv_chr1_Sto4_2_16_F	TATCGCTTCCGGACAATCGTT
Bv_chr1_Sto4_2_16_R	CTGGGGCCTACTTAAGCCTTT
Bv_chr1_Sto4_2_19_F	CTCCTACTCTCTCTGTCTTTGCAT
Bv_chr1_Sto4_2_19_R	TCTGCTCTTTCGCTCGTAACC
Bv_chr2_Sto4_2_22_F	CACTGGGAGCAACTCACGAT
Bv_chr2_Sto4_2_22_R	ACTCTTATTGGTGTGCCATCTACA
Bv_chr2_Sto4_2_25_F	TGGGCTAATTTCGTTGCCGT
Bv_chr2_Sto4_2_25_R	CCTCAGTCTCAGTGCCTCAC
Bv_chr2_Sto4_2_27_F	CTGATTCCATGGCCTGGTCC
Bv_chr2_Sto4_2_27_R	TCATGTCACTGAGACGAGAAACA
Bv_chr2_Sto4_2_33_F	TAACACCGGTGCTCAGGCTA
Bv_chr2_Sto4_2_33_R	TCACCCAATAATGCCTCCGTG
Bv_chr3_Sto4_2_39_F	GAGTGAAAACGAGGTCGGAGT
Bv_chr3_Sto4_2_39_R	TCGTAACTCCCCTGTCTATGG
Bv_chr3_Sto4_2_42_F	GAGCCACCATTAAGCCCGT
Bv_chr3_Sto4_2_42_R	CCACTCCTGGAGCTTTAGTGG
Bv_chr3_Sto4_2_43_F	CTCACTCCCTGCTATCAAGCG

Bv_chr3_Sto4_2_43_R	TTGGGATCAGCTGCTTAGGA
Bv_chr3_Sto4_2_47_F	CCGCTACACTGGATGTACCC
Bv_chr3_Sto4_2_47_R	GACCTCGCACCACTACGTTT
Bv_chr3_Sto4_2_50_F	CGAGAACACGCTTGTGACCA
Bv_chr3_Sto4_2_50_R	GGTGTGCTCCGTGCAGAATA
Bv_chr3_Sto4_2_51_F	ATGGTCGCGATCAAGCCATC
Bv_chr3_Sto4_2_51_R	GTGGCGTAAGCACTTGGAAAA
Bv_chr3_Sto4_2_54_F	TGGCCGAAAAATGCAATGGT
Bv_chr3_Sto4_2_54_R	AAAGCCGCCACCACAAAAAG
Bv_chr4_Sto4_2_79_F	TCGGCATTACCTCTTGATTCCC
Bv_chr4_Sto4_2_79_R	AAAGTGTGTTGTTTCCCCCA
Bv_chr5_Sto4_2_81_F	TCCTGCTTTGGTAGCTCGG
Bv_chr5_Sto4_2_81_R	GTTCCTCCGGGCTTCAGATTC

3.4 PCR Analysis

For PCR reactions the master mix was prepared for a total of 13 samples with 12 samples for each DNA and one sample as an empty master mix (Table 4). After adding 9 µL of master mix into each PCR tube, 1 µL of each DNA sample was added to each tube and then centrifuged for a few seconds for proper mixing of DNA with the master mix. PCRs were performed in an Eppendorf Thermo cycler Nexus Gradient. This is depicted in Table 3.

Table 3: PCR protocol followed for microsatellite primers

STEPS	TEMPERATURE	TIME	CYCLES
1.PRE DENATURATION	95°C	2 minutes	30 cycles
2. DENATURATION	95°C	15 seconds	
3. ANNEALING*	60°C	15 seconds	
4. EXTENSION	68°C	1 minute	
5. FINAL EXTENSION	68	1 minute	
6. PCR TEMPERATURE	10	Constant temperature	

Table 4. Protocol for PCR master mix.

	For one sample	**For 13 samples**
Water	6.65µl	86.45µl
(Green) dream Taq buffer	1µl	13µl
Forward primer	0.5µl	6.5µl
Reverse primer	0.5µl	6.5µl
dNTPs	0.25µl	3.25µl
Dream Taq polymerase	0.1µl	1.3µl

3.5 Gel Electrophoresis

a. Buffer preparation: For gel electrophoresis 1X TBE (Tris base) buffer with pH 8.2- 8.4 was prepared from 5x TBE (4:1 ratio).

b. Gel preparation: The concentration of gel is 1 % with agarose. After preparation of the buffer 300 ml of the buffer is added to a conical flask and add 3 grams of agarose for 1% gel concentration. After keeping the gel solution in the oven for two to three minutes get clear gel without any lumps and cool it down under tap water and add Midori stain of 12 µL, into the flask and stir it well. The gel was poured onto the frame, which was already mounted with combs and waited for one hour to solidify the gel for loading.

c. DNA Loading: After completion of PCR, amplified DNA samples were loaded in the wells of the gel along with a 1 kb DNA ladder and kept for gel electrophoresis.

d. Gel electrophoresis: Gel run for electrophoresis at 120v, 300 current, and for 60 minutes. After gel electrophoresis, the images of gels were taken, by visualizing under ultraviolet light. The 1kb DNA ladder is also visualized and the length of DNA bands can be estimated by using 1kb ladder as a reference.

3.6 Statistical data analysis

The ILP marker scoring was done manually. By examining the electrophoretic images of the DNA bands, the length of each band of all the primers was counted based on the ladder length and data accumulated. The codominant marker matrix with diploid individuals was created and used in GenALEx 6.5 (Peakall and Smouse, 2006) for creating a genetic distance matrix and analysis of molecular variance (AMOVA) and principal coordinate analysis (PCoA).

Genetic distance: Genetic distance is a measure of the genetic divergence between species or between populations within a species, whether the distance measures the time from a common ancestor or the degree of differentiation. Populations with many similar alleles have lesser genetic distance. GenALEx 6.5 was used to estimate genetic distance.

Analysis of Molecular Variance: AMOVA is a statistical model used for the molecular algorithm in a single species, typically biological. AMOVA is used to calculate the level of genetic differentiation among different populations. It uses molecular markers and tells us the difference between populations and within populations. It was performed to evaluate differentiation among the three subpopulations.

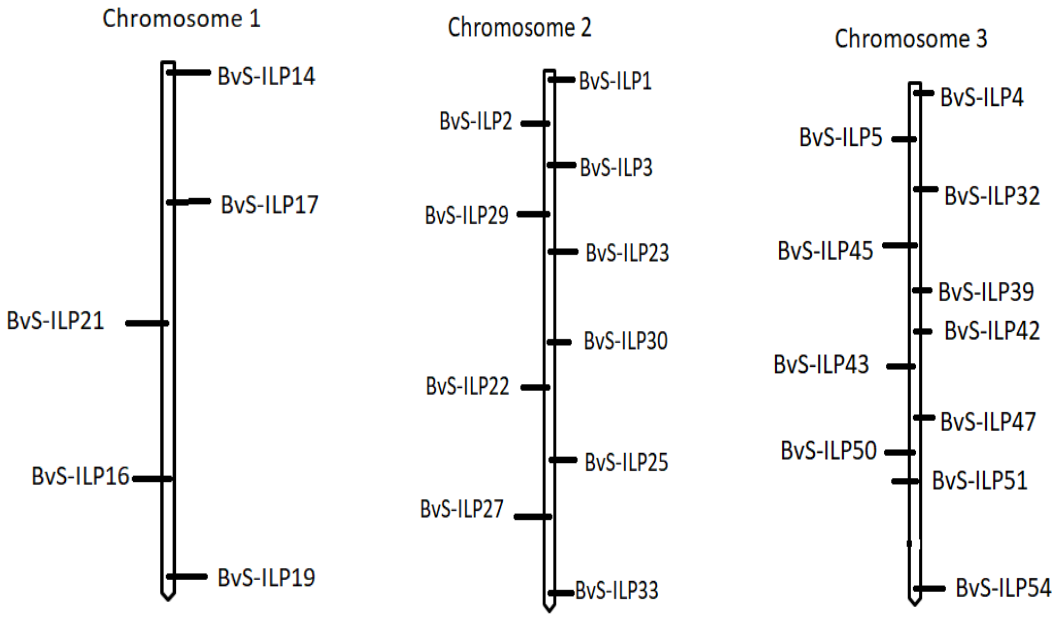
Principal Coordinates Analysis: PCoA is a multivariate analysis method that lets you analyze a proximity matrix, whether it is a dissimilarity matrix or a similarity matrix. It was performed to visualize the genetic diversity of the studied accessions.

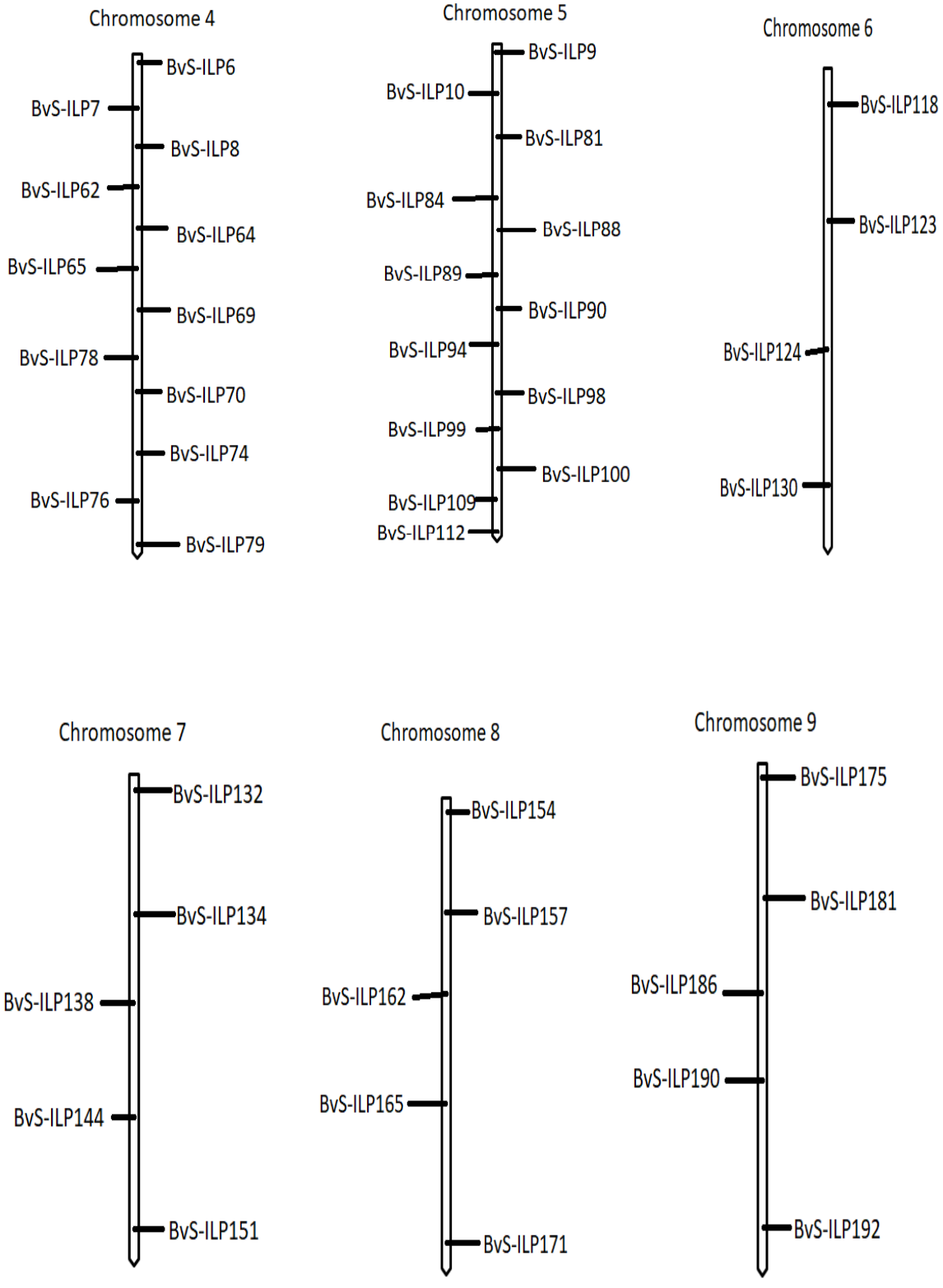
4. Result

4.1 Development and validation of the candidate ILP Markers

Insertion sites of 70 *BvSto* MITEs within introns of annotated genes were chosen to develop *Beta vulgaris Stowaway*-like Intron Length Polymorphism (BvS-ILP) markers evenly distributed throughout the genome (figure 1).

Figure 1: Physical genome distribution of the 70 developed BvS-ILP markers on nine chromosomes of the sugar beet genome. The vertical bars correspond to the position of introns harbouring *BvSto* insertions, selected for the development of ILP markers.




Chromosome 4
BvS-ILP6
BvS-ILP7
BvS-ILP8
BvS-ILP62
BvS-ILP64
BvS-ILP65
BvS-ILP69
BvS-ILP78
BvS-ILP70
BvS-ILP74
BvS-ILP76
BvS-ILP79
Chromosome 5
BvS-ILP9
BvS-ILP10
BvS-ILP81
BvS-ILP84
BvS-ILP88
BvS-ILP89
BvS-ILP90
BvS-ILP94
BvS-ILP98
BvS-ILP99
BvS-ILP100
BvS-ILP109
BvS-ILP112
Chromosome 6
BvS-ILP118
BvS-ILP123
BvS-ILP124
BvS-ILP130
Chromosome 7
BvS-ILP132
BvS-ILP134
BvS-ILP138
BvS-ILP144
BvS-ILP151
Chromosome 8
BvS-ILP154
BvS-ILP157
BvS-ILP162
BvS-ILP165
BvS-ILP171
Chromosome 9
BvS-ILP175
BvS-ILP181
BvS-ILP186
BvS-ILP190
BvS-ILP192

A total of 70 pairs of primers were used with 12 DNA samples for the study. Thirty-five pairs got monomorphic, 32 pairs are polymorphic and the remaining 3 pairs got no amplification (Table 5). No amplification may be due either to DNA insertions or deletions or to not efficient primers in PCR reactions . The number of *BvSto* insertion sites evaluated per chromosome varied from 11 (chromosome 5) to 4 (chromosome 6).

Table 5: No. of primers used and the no. of got amplification:

	Number
Primers	70 pairs
Monomorphic	35
Polymorphic	32
No amplification	3

Upon PCR amplification, 32 of the 70 sites showed the expected *BvSto* insertion-based polymorphisms; however, in the case of 7 sites, an additional amplification product was present in at least one accession. In the remaining 38 sites, 3 do not amplify efficiently, and 35 were monomorphic for all tested cultivars (Table 6). Out of 70 insertion sites, most of the insertions are present on chromosome 5 with 13 insertion sites and less on chromosome 6 with just 4 insertions. A greater number of polymorphisms were observed on chromosome 5 with 6 polymorphisms.

Table 6: Results of the experimental validation of developed candidate BvS-ILP markers.

Chromosome	**No of insertion sites**	**Polymorphic with two allelic variants resulting from *BvSto* insertion**	**Polymorphic with two allelic variants resulting from *BvSto* insertion and an additional variant**	**Monomorphic**	**No amplification**
1	5	2	-	3	-
2	10	3	1	5	1
3	11	2	1	8	-
4	12	4	1	7	-

5	13	4	2	6	1
6	4	3	-	1	-
7	5	3	-	2	-
8	5	3	-	1	1
9	5	1	2	2	-
Total	**70**	**25**	**7**	**35**	**3**

In *BvSto* insertions, the length of introns varied from 400 to 2150 bp. Based on the length of the introns after amplification, the markers were divided into six classes i.e. I, II, III, IV V classes from 400 to 1400bp, each at 200 bp interval, and class VI is greater than 1400 (Table 7). Class II, III, and IV markers were most abundant with 76.1%, which are successfully amplified indicating the most suitable length of introns considered for BvSto-ILP markers. Class I are short in length, meaning that no MITEs are present in these markers. Class VI is 1400 bp longer than the polymorphic length, which means that they have additional insertions. Class III (800-1000) showed the highest percentage 84.2% of successful amplification rate, indicating that this is the most suitable length of introns considered for ILP markers. In V and VI classes there is no successful amplification, so we did not consider this class for further studies.

Table 7: The intron length-based classification of BvS-ILP markers.

Class	**The range of intron length[bp]**	**Number of candidates' BvS-ILP markers**	**Positively validated BvS-ILP markers**
I	400-600	7	3
II	600-800	17	7
III	800-1000	19	16
IV	1000-1200	15	6
V	1200-1400	6	0
VI	>1400	3	0

4.2 Amplification of BvS-ILP markers in 12 beet cultivars:

Table 8: Zygosity of each genotype for specific polymorphic markers

***H/O- Heterozygous occupied, H/Adv-Heterozygous additional variant, h/O-Homozygous occupied, h/E- Homozygous empty, h/Adv- Homozygous additional variant.**

MARKERS	GENOTYPES											
	SB1	**SB2**	**SB3**	**SB4**	**SB5**	**SB6**	**SB7**	**SB8**	**SB9**	**SB10**	**FB1**	**FB2**
BvS-ILP21	h/O	h/E	h/E	h/O	h/E	h/E	h/O	h/E	h/E	h/E	h/E	H/O
BvS-ILP19	H/O	h/E	h/E	h/E	h/E	h/E	h/E	h/E	h/E	h/O	h/O	h/E
BvS-ILP1	h/E	h/O	h/O	h/O	H/O	h/E	h/E	h/E	h/E	h/E	h/E	h/E
BvS-ILP3	H/O	H/O	h/O	h/E	h/E	h/E	h/E	h/E	h/E	h/E	h/E	h/E
BvS-ILP30	H/Adv	h/E	h/E	h/E	H/Adv	h/E	H/O	H/O	H/O	h/E	h/E	h/E
BvS-ILP25		h/E	h/E	h/E	h/O	H/O	h/O			h/E	h/O	H/O
BvS-ILP5	h/O	h/O	h/O	h/O	H/Adv	H/O	h/O	h/O	h/O	h/E	H/O	h/O
BvS-ILP43	h/E	h/O	h/O	h/E	h/E	h/E	h/E	h/E	h/E	h/O	H/O	H/O
BvS-ILP54	h/E	h/E	h/E	H/O	h/E	h/E	h/E	h/E	h/E	h/E	h/E	h/E
BvS-ILP7	h/E	H/O	h/E	h/O	h/E	h/O	h/E	h/E	h/O	h/E	h/E	h/E
BvS-ILP8	h/O	h/O	h/O	h/E	h/O	h/O	h/O	h/O	h/O	h/O	h/O	h/O
BvS-ILP64	h/E	h/E	h/E	h/O	h/E	h/E	h/E	h/E	h/E	h/E	h/E	h/E
BvS-ILP65	h/O	h/O	h/O	h/O	h/O	h/O	h/E	h/E	h/E	h/E	h/O	h/O
BvS-ILP76	H/O	h/O	h/O	h/O	h/E	h/Adv	h/O	h/O	h/O	h/O	h/E	H/O
BvS-ILP9	H/O		h/O	h/E	h/E	h/E	h/O	h/O	h/O	h/O	h/E	h/O
BvS-ILP90	h/E	h/E	h/E	h/E	h/O	h/E	h/E	h/E	h/E	h/E	h/E	h/E
BvS-ILP99	h/E	H/Adv	h/O	h/E	H/Adv	h/Adv	h/E	h/E	h/E	h/E	h/E	H/O
BvS-ILP112	H/O	H/O	h/O	h/O	h/O	h/O	h/O	h/O	h/O	h/O	H/O	H/O
BvS-ILP84	h/E	h/E	h/E	h/O			h/E	h/E	h/E	h/E	h/E	h/E
BvS-ILP88	h/O	h/O	h/E	h/O	h/Adv	h/O	h/E	h/E	h/E	h/E	H/Adv	h/O
BvS-ILP123	h/E	h/O	H/O	h/E	h/E	h/E	h/E	h/E	h/E	h/E	h/E	h/E
BvS-ILP124	h/O	h/E	h/E	h/E	h/E	h/E	h/O	h/E	h/E	h/E	h/E	H/O
BvS-ILP130	h/O	H/O	h/E	h/E	h/E	h/O	h/E	h/O	h/O	h/E	h/O	h/E

BvS-ILP165	h/O	h/O	h/O	h/O	h/O	h/E	h/O	h/O	h/O	h/O	H/O	H/O
BvS-ILP171	h/E	h/E		h/O	h/O	h/O	h/E	h/E	h/E	h/E	h/O	h/E
BvS-ILP154	h/O	H/O	h/E	h/O	h/O	h/O	h/O	h/O	h/O	h/E	h/E	h/E
BvS-ILP151	h/O	H/O	h/O	h/O	h/O	h/O	h/O		h/O	h/O	h/E	H/O
BvS-ILP132	h/O	h/O	h/O	h/O	h/O	h/O	h/O	h/E	h/E	h/O	h/E	h/E
BvS-ILP134	H/O	h/O	h/O	h/E	h/O	H/O	h/E	h/E	h/E	h/O	h/E	h/E
BvS-ILP181	h/E	h/E	h/E	h/O	h/O	h/E	h/Adv	h/Adv	h/Adv	h/Adv	h/E	H/O
BvS-ILP175	h/O	h/E	h/E	h/E	h/E	H/O	h/E	h/E	h/E	h/E	h/E	h/E
BvS-ILP190	h/O	H/O	h/O	h/O	h/Adv	h/O	h/E	h/E	h/E	h/E	h/O	H/Adv

Amplification of BvS-ILP 79th, 130th, 21st, 154th, 30th, 88th, 157th, and 33rd pair markers in 12 sugar beet cultivars images are given below. Beet accessions from 1 to 12 are listed in the following images. Amplification of two alleles corresponding to empty is marked as (A), occupied is marked as (B), and amplification of additional insertion is marked as (C) resulting from an unclassified rearrangement within the introns. 1 kb DNA ladder is marked as (M). The size range of the ladder is 250bp to 10,000 bp. The DNA ladder consists of 14 DNA fragments.

a. Amplification of the BvS-ILP130th marker is polymorphic (Figure 2). It has both homozygous and heterozygous accessions. Lane 2 is heterozygous. All remaining lanes are homozygous. MITEs are present in 1,6,8,9 and 11 lanes but absent in 3,4,5,7,10 and 12 lanes. BvS-ILP130th marker belongs to chromosome 6th of *Beta vulgaris*.

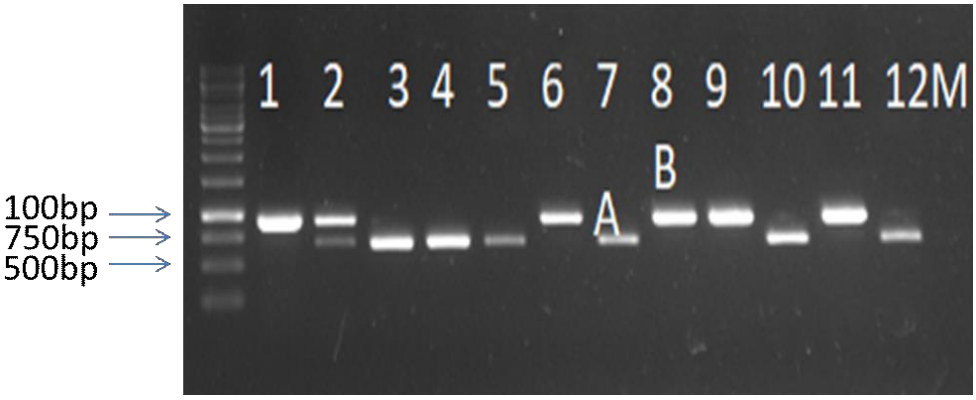


Fig 2: Electrophoretic image of BvS-ILP 130th marker

b. Amplification of BvS-ILP21st marker in 12 sugar beet cultivars identified polymorphisms (Figure 3). Lane 12th is heterozygous while all remaining lanes are homozygous. Lanes 1, 4, 7 and 12 lanes show MITEs. Lanes 2,3,5,6,8,9,1 and 11 did not show any MITE. BvS-ILP21st marker belongs to chromosome 1 of *Beta vulgaris*.

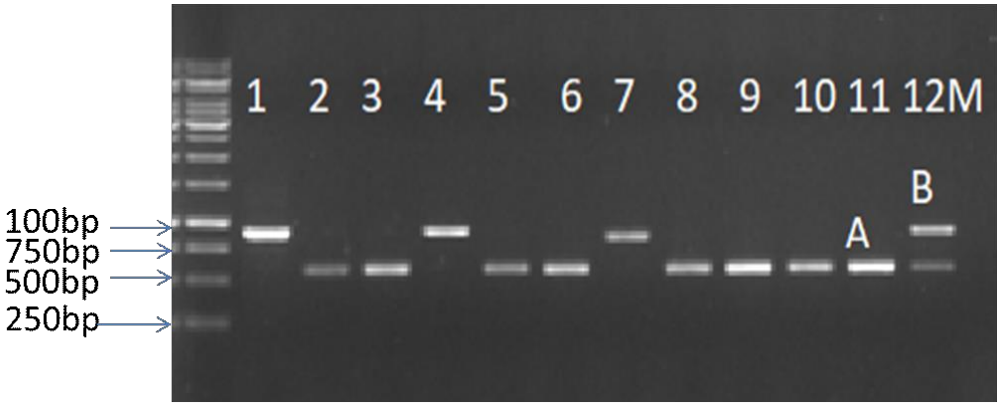


Fig 3: Electrophoretic image of BvS-ILP 21st marker

c. BvS-ILP154th marker is polymorphic (Figure 4). In the 2nd lane, this marker is heterozygous, while in all remaining lanes it is homozygous. Lane 2 shows MITE presence. Lanes 1,4,5,6,7,8 and 9 are homozygous and show MITEs presence, but lanes 3,10,11 and 12 are homozygous and do not show any MITE. BvS-ILP54th marker belongs to chromosome 8th of *Beta vulgaris*.

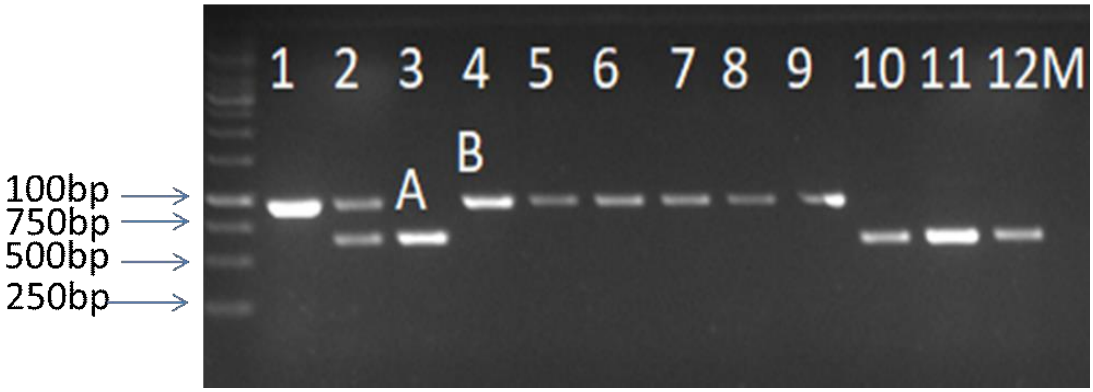


Fig 4: Electrophoretic image of BvS-ILP 154th marker

d. BvS-ILP30th marker is polymorphic (Figure 5). In the 1,5,7,8 and 9 lanes, this marker is heterozygous: lanes 7,8 and 9 are occupied with MITE and lanes 1 and 5 show some additional insertions other than MITEs. Lanes 2,3,4,6,10,11 and 12 are homozygous and do not show any MITE. BvS-ILP30th marker belongs to chromosome 2nd of *Beta vulgaris*.

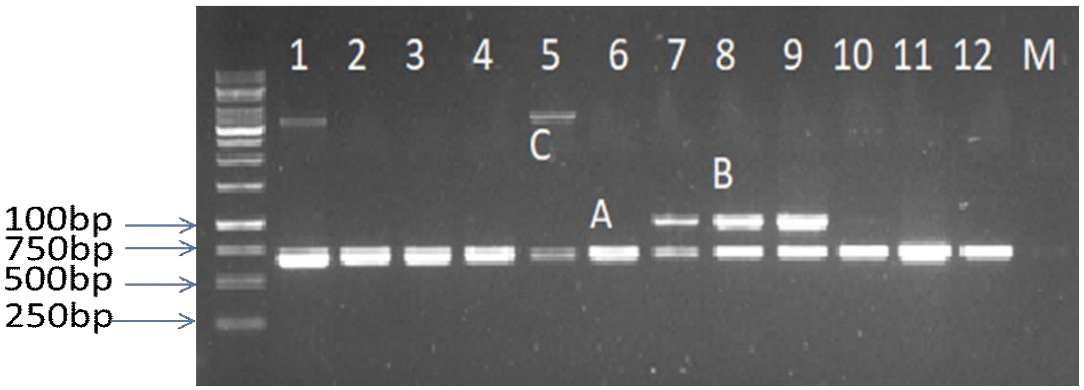


Fig 5: Electrophoretic image of BvS-ILP 30th marker

e. BvS-ILP88th marker is polymorphic (Figure 6). In lane 12th the plant is heterozygous. All remaining lanes it is homozygous. Lanes 4,5 and 12 show MITE. Lanes 7,8,9 and 10 show some additional insertions other than MITE. Lanes 1,2,3,6 and 11th do not show any MITE. BvS-ILP88th marker belongs to chromosome 5th of Beta vulgaris.

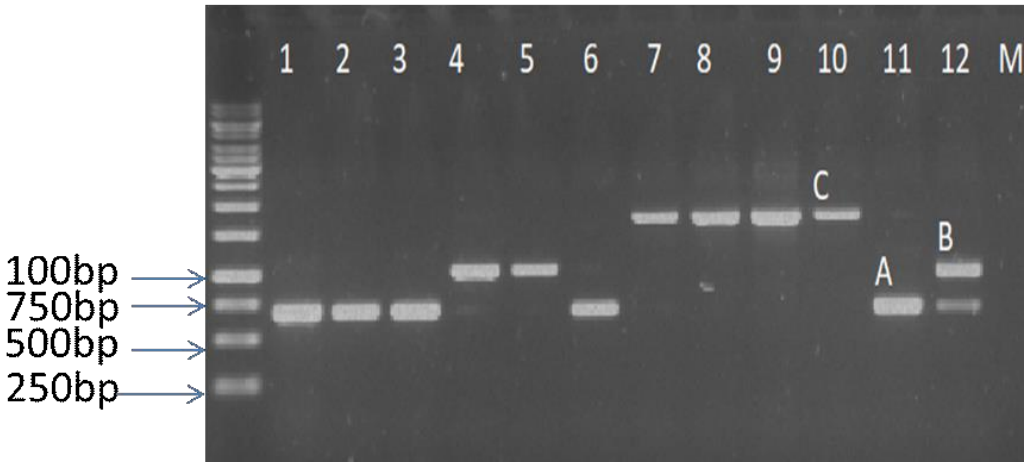


Fig 6: Electrophoretic image of BvS-ILP 88th marker

f. Amplification of the BvS-ILP79th marker is monomorphic (Figure 7). It seems to be the effect of amplification derived from two paralogs, one bearing the insertion and one empty. BvS-ILP79th marker belongs to chromosome 4th of *Beta vulgaris*.

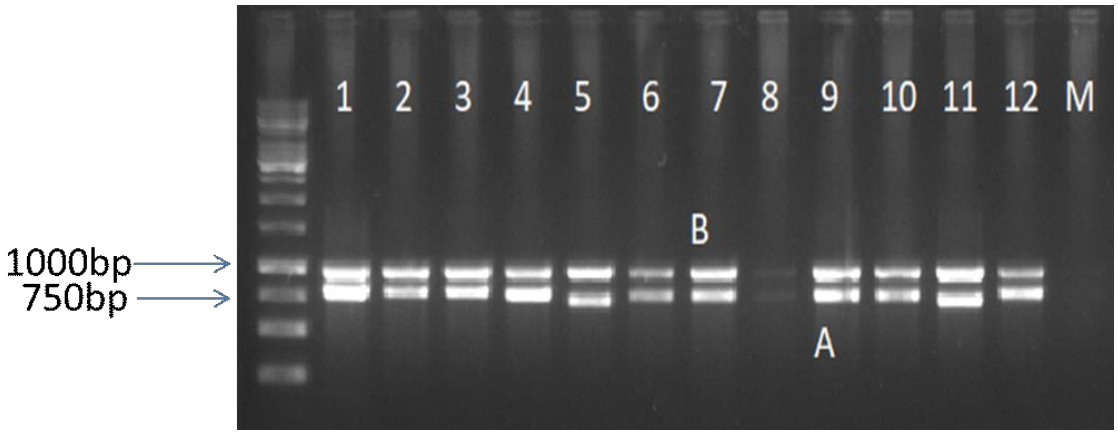


Fig 7:Electrophoretic image of BvS-ILP 79th marker

g. BvS-ILP157th marker is monomorphic. It is homozygous at all lanes. It does not show any MITE in those cultivars. BvS-ILP157th marker belongs to chromosome 8th of Beta vulgaris.

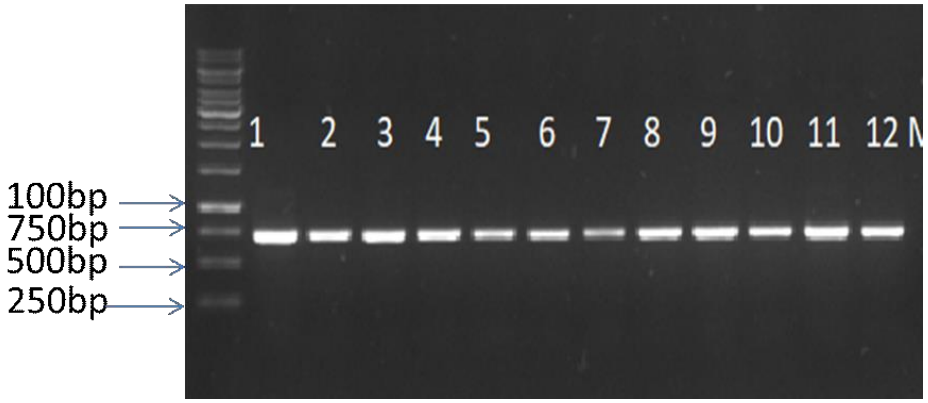


Fig 8: Electrophoretic image of BvS-ILP 157th marker

h. BvS-ILP33 marker got no amplification (Figure 9). This may be due to the primers which are not working for the DNA.

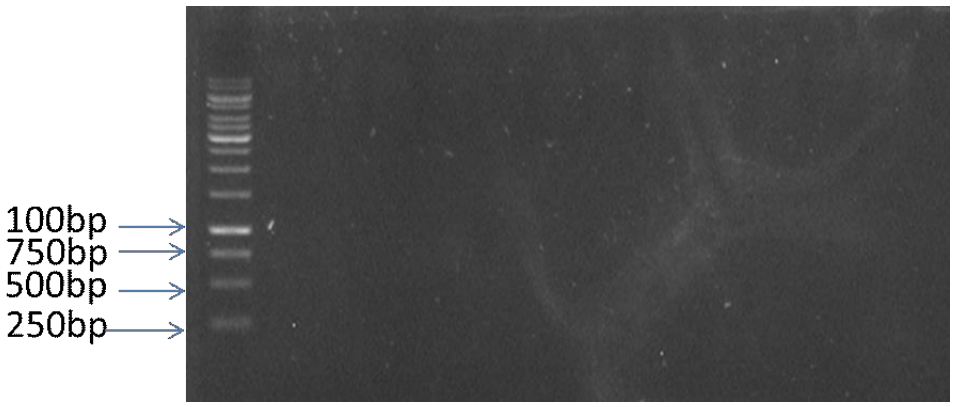


Fig 9: Electrophoretic image of BvS-ILP 33rd marker

4.3 Assessment of genetic diversity:

4.3.1 Genetic distance

The genetic distance (GD) is one of the important measures to understand the diversity of the parents. GD should be optimum to exploit the heterosis segregation in crop breeding. Genetic distance measures the accumulated allelic differences per locus. The highest genetic distance is between SB4 and SB10 which is 102. And the lowest genetic distance is between FB1 and FB2 (Table 9).

SB1	SB2	SB3	SB4	SB5	SB6	SB7	SB8	SB9	SB10	FB1	FB2	
0	61	71	72	70	67	75	78	70	90	67	60	SB1
61	0	44	69	66	68	71	74	66	67	64	63	SB2
71	44	0	87	67	90	75	82	74	57	84	73	SB3
72	69	87	0	68	67	94	95	87	102	89	90	SB4
70	66	67	68	0	50	63	82	74	68	60	61	SB5
67	68	90	67	50	0	89	82	74	85	61	78	SB6
75	71	75	94	63	89	0	41	41	54	77	60	SB7
78	74	82	95	82	82	41	0	8	49	76	65	SB8
70	66	74	87	74	74	41	8	0	41	68	57	SB9
90	67	57	102	68	85	54	49	41	0	77	68	SB10

67	64	84	89	60	61	77	76	68	77	0	35	FB1
60	63	73	90	61	78	60	65	57	68	35	0	FB2

Table: 9 Results of genetic distance *SB (Sugar beet), *FB (Fodder beet)

4.3.2 AMOVA:

AMOVA stands for Analysis of Molecular Variance and is a method used to detect population differentiation utilizing molecular markers. Probability values were estimated by 999 permutations to determine whether the partitioning of variance components was significant. For calculating AMOVA the whole population was divided into three smaller populations: Population I is sugar beet multigerm, population II is sugar beet monogerm and the third population belonged to fodder beet. The estimated variance among populations is 25%, among individuals is 53%, and within individuals is 22% (Figure 10).

Pop	Pop1	Pop2	Pop3		
n	12	8	4		
SSWP	100.250	33.250	17.250		
Summary AMOVA Table					
Source	df	SS	MS	Est. Var.	%
Among Pops	2	67.167	33.583	2.720	25%
Among Indiv	9	122.750	13.639	5.653	53%
Within Indiv	12	28.000	2.333	2.333	22%
Total	23	217.917		10.706	100%

Fig: 10 AMOVA result by using GenALEx 6.5

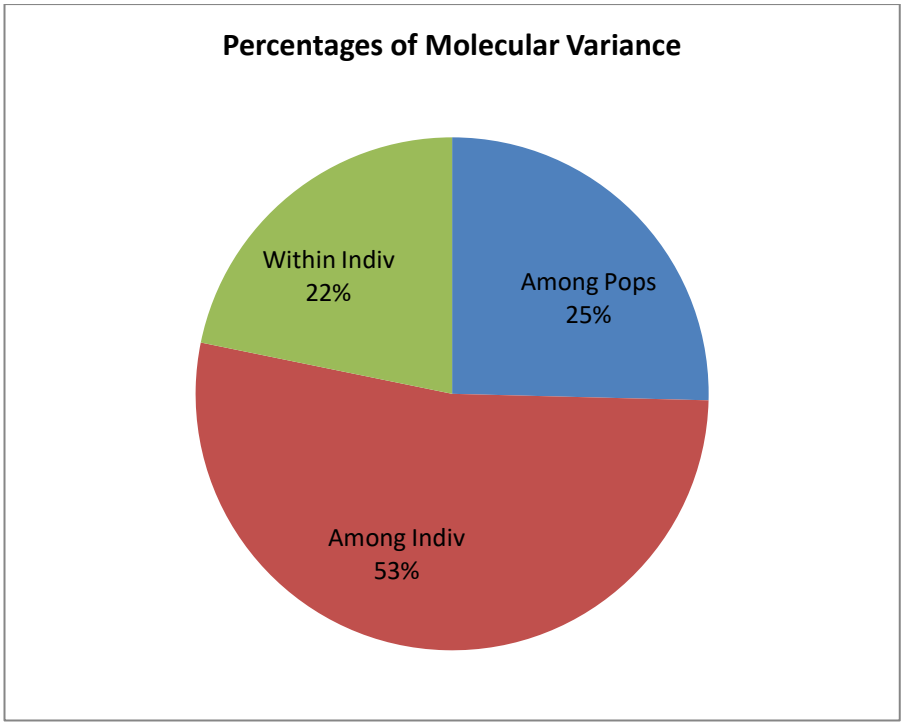


Fig: 11 Percentages of molecular variance

4.3.3 Principal Coordinates Analysis (PCoA):

PCoA shows a graphical representation of different population diversity and the differences and similarities among individual populations. The diversity of the 12 cultivated species was revealed by PCoA, their distribution is depicted in figure 12. The percentage of variation is explained by the first 3 axes, 1st axis got 23.88%, 2nd axis got 15.07% and 3rd axis got 13.84%. In this analysis, highly correlated samples are formed in clusters. So the cultivars ordinated closer to one another are more similar than those ordinated further away. A clear separation of fodder beet and sugar beet accessions can be seen along coordinate II, while multigerm and monogerm accessions are separated along coordinate I.

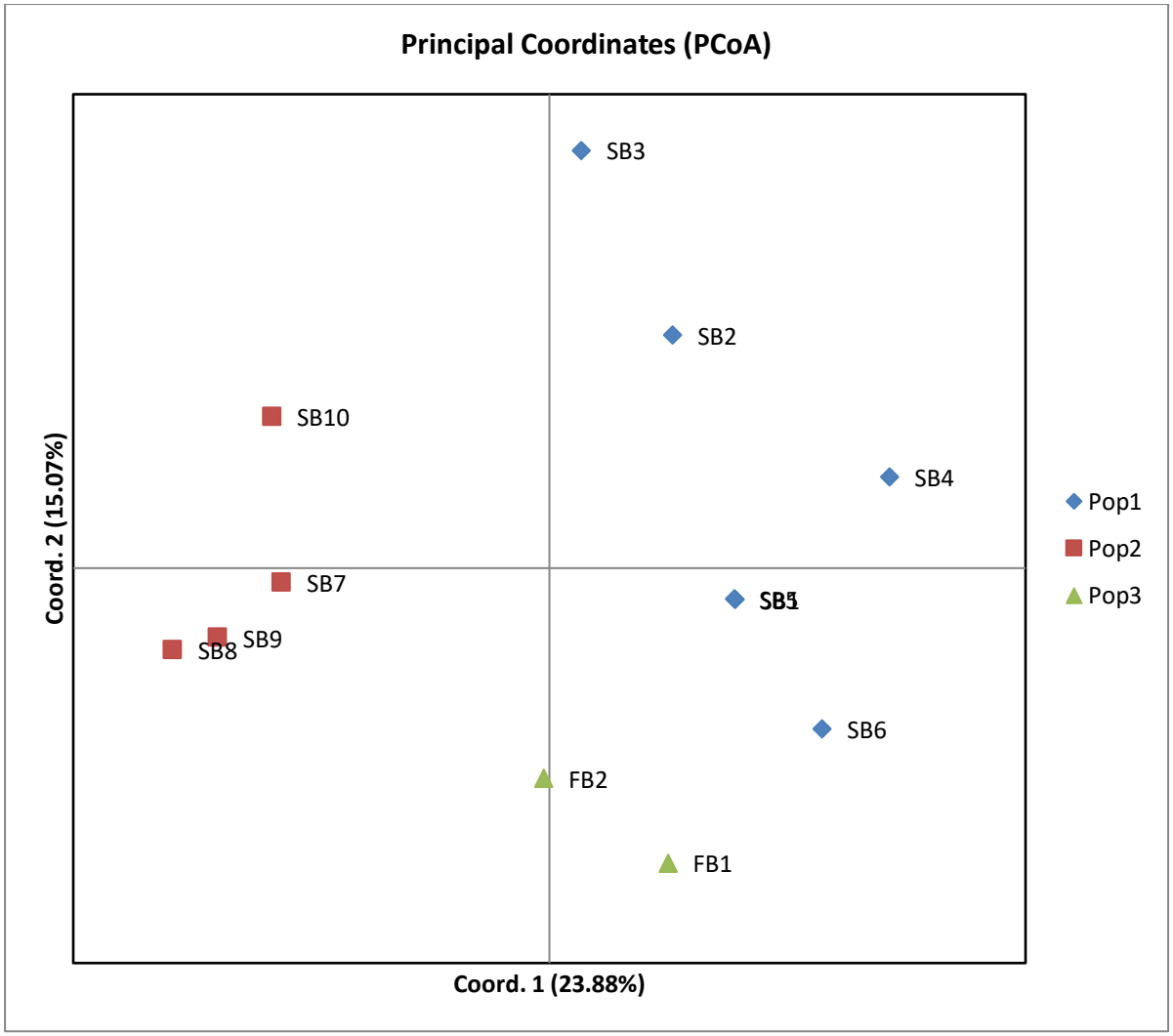


Figure 12: Principal coordinates analysis

5. Discussion:

In sugar beet, MITEs can reside anywhere in the non-coding portion of the genome, but in this experiment we were specially targeting copies residing in introns, as it is easy to design primers anchored in exons flanking introns. Exons are usually more conserved than non-coding regions, thus intron polymorphisms are expected to be more reproducible than random ones.so all these factors lead to the sugar beet crop being highly heterozygous and polymorphic. Sugar beet genetic diversity is also very high. The parental lines which can be

used to get the F1 hybrid must be homozygous and the F1 hybrid cultivars must be heterozygous in order to get warrant uniformity of the cultivar.

The two largest MITE families are *Stowaways* and *Tourists*. The *Stowaway* family is identified as a member of the *Tc1/Mariner* superfamily (Grzebelus, 2018). In this study, a total of 12 cultivars are used and 70 pairs of primers were tested in PCR reaction in order to find out intron length polymorphisms (ILPs). PCR protocols allowed the generation of long fragments, which in turn facilitated the finding of regions flanking *Stowaway* insertions. Using agarose gels for the separation of the amplicons simplified the detection of polymorphisms. In all 12 samples, amplification with tested ILP markers resulted in PCR products that varied in size and number.

In our work 45.71% of markers got polymorphism and 50% of primers got monomorphic of 70 markers. In similar studies carries out on sugar beet using RAPD markers (Ghasemi et al., 2014), the average polymorphism observed for the 10 primers used for the study is 82.33%. BvS-ILP markers are codominant, which means both alleles are expressed when co-occurring in an individual. Therefore, with codominant markers, heterozygotes can be distinguished from homozygotes, allowing the determination of genotypes and allele frequencies at loci. RAPD and ISSR are dominant markers, which can identify only a single dominant allele. The level of polymorphism observed by ISSR markers is 97.2% and for RAPDs are 93% (Izzatullayeva et al., 2014).

In this study out of 12 genotypes, most of the heterozygous alleles, occupied with MITEs were observed in the SB2 genotype, which is 8 out of 32 polymorphic markers, and the FB2 genotype, which is 11 out of 32 polymorphic markers. In the remaining genotypes, most of the markers are monomorphic alleles that are occupied with MITE. A result reported by (Taški-Ajduković et al., 2017) suggests that in total, 40 SSR loci had 129 different alleles. In this experiment we generally expected a strictly biallelic inheritance, but occasional off-type variants (i.e. there were more than two alleles for certain loci) also appeared. The number of alleles varied from 2 to 5, with an average of 3.22 per locus, which is in agreement with the results of (Fugate et al., 2014).

In this study, the genetic distance calculated between the three populations is very high. The highest distance is between SB4 and SB10, which is 102. The lowest genetic distance is

between FB1 and FB2, which is 35. The average genetic distance between all the cultivars in the three populations is 63.30.

The AMOVA indicates that the estimated variance among populations is 25%, among individuals is 53%, and within individuals is 22%. This is in agreement with similar studies on cultivated sugar beet by Abbasi et al. (2014). Also, similar experiments conducted by (Wang & Goldman, 1999) on genetic distance and diversity of different accessions of table beet and sugar beet got similar results, where the populations were broadly scattered in the MSD plot and showed the most significant variation within each population. All these results indicate that there is a significant amount of genetic diversity within and among each population of beet population.

The results of the principal coordinate analysis (PCoA) revealed the genetic diversity of the three populations by using ILP markers. The first two principal coordinates explained 23.88% and 15.07% of the molecular variance. By using SSR markers the PCoA based on MRD estimates between all pairs of sugar beet inbreds, the first two principal coordinates explained 17.6% and 7.6% of the molecular variance (J. Li et al., 2010).

In all 32 Stowaway inserted polymorphic markers, a minimum of 2 to 4 polymorphisms were observed in every marker of the sugar beet. The result reported by Grzebelus et al., 2011 shows that the identified intraspecific insertion polymorphism suggests that at least one of the VulMITE families was active in the Betoideae subspecies, which are obtained and can be used to confirm homozygosity during the production of double haploids. Double haploids save time and money compared to conventional breeding because double haploids have fewer crossing-overs and have a greater chance of inheriting favourable traits. Integrating marker-assisted selection with double haploids allows for rapidly producing pure lines.

In this overall experiment as we used ILP markers for the study, which is a unique and novel technique for marker-assisted selections and takes less time and cost efficiency to confirm homozygosity, which will be useful for further breeding programs during the production of double haploids.

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