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ASSESSMENT OF GENETIC DIVERSITY WITHIN GERMPLASM ACCESSIONS IN TOMATO USING MORPHOLOGICAL AND MOLECULAR MARKERS

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ABSTRACT

A total of 25 high yielding tomato accessions were selected for the characterization that helped in the reliable varietal selection programme for breeding. All tomato accessions were analyzed by two parameters e.g. morphological and molecular parameters. For morphological characterization the plant height, shape, size, leaf length and width, and fruit size, colour and shape were selected and for molecular characterization RAPD markers were used. A total 25 RAPD decamer primers were selected for the genetic analysis of all tomato accessions. Only 15 polymorphic RAPD primers were accessed for the genetic distance calculation to find out the phylogenetic relationship among 25 tomato accessions under study. A total of 130 loci were generated outof which 98 were polymorphic by 15 primers with 05-14 loci/primer having fragment's size range from 400to 2500bp maximum. The Nie and Lie's Coefficients was used to calculate the genetic similarity. The extent of genetic diversity and construction of phylogenetic tree was done by DNAMANN software. The average genetic similarity observed across all the genotypes was 75.6% with 24.4% polymorphismin 25 tomato accessions. Although RAPD study supports the morphological characters but not upto 100%.

Key words: Randomly amplified polymorphic DNA, Polymerase chain reaction, *Lycopersiconesculentum.*

INTRODUCTION

Tomato is an herbaceous plant belongs to family Solanaceae or Nightshade family. It is one of the significant vegetable crops of special economic importance in the horticultural industry, originating in South America and its many varieties are now commonly grown in greenhouses in cooler climates(He *et al.,*2003).Itis the most popular garden vegetable belongs to the genus *Lycopersicon*, the resemblance between leaves and flowers of potato and tomato plants seems to certify this taxonomic grouping (Wang *et al.,* 2005 and Shidfar *et al.,* 2011).

The popularity of tomato and its products continue to rise as it is a good source of vitamin A and C in significant amount. It is extensively used in salad as well as for culinary purposes and also used for various processed formsinclude pastes, sauces, pulps, juices, ketchup and as flavoringingredients in soups, meat or fish dishes(Gosselin and Trudel, 1984).The fruit contains significant amounts of lycopene, beta-carotene, magnesium, iron, phosphorus, potassium, riboflavin, niacin, sodium and thiamine. It has antioxidant properties and potential beneficial health effects (Zhang *et al.,* 2009). Tomato intake is reduced entire cholesterol, LDL cholesterol and triglyceride levels in white blood cells, reducing cardiovascular risk related with type 2 diabetes also decreased risk of breast cancer, neck cancers and strongly protective against neurodegenerative diseases (Freedman*et al.,* 2008).

Tomato is grown worldwide for its edible fruitshaving bisexual flowers, although often grown outdoors intemperateclimates as they do not tolerate frost.The tomato species cultivate as annuals in colder regions while they are perennial in warmer regions, it is a self-pollinated crop but in some cases as high as 30% cross-pollination. The plant has compound leaves, made up of leaflets which are arranged along with 2 to 6 opposite or sub-opposite pairs of petiolate and subsessileleaflets. (Peralta *et al.,* 2005).Tomato cultivars produce red, yellow, pink, green, black or white fruit and they have been selected with varying fruit typesand for optimum growth in differing growing conditions. The plants normally grow to 1–3 meters (3–10 ft) in height and having slender and herbaceous but weak stem that sprawls over the ground and vines over other plants.(Rico-Garcia *et al.,* 2009).

Tomato is the well-studied crop species forbreeding, genetics and genomics. It is one of the initial crop plants for which a genetic linkage map was constructed, presently there are several molecular maps based on crosses between the cultivars and many wild species of tomato. Genetic analysis of tomato is essential to enhance the genetic yield potential and maximum utilization of the desirable characters for synthesizing of any ideal genotypes (Kumar *et al.,*2003).The assessment of genetic diversity within and between populations of tomato varieties is measuredby using morphological, biochemical and molecular characterization (Garcia *et al.,*2004).

Morphological markers have several defacts that reduce the ability to estimate genetic diversity in plants as

it highly dependent on the environment for expression. Molecular markers can give an effective tool for efficient selection of desired agronomic traits because theyare based on the plant genotypes and also are independent of environmental variation (Franco*et al.,* 2001). Researchers have been calculated genetic variation in tomato landrace and cultivar collections using several molecular techniquesincluding amplified fragment length polymorphism (AFLP), restricted fragment length polymorphism (RFLP), simple sequence repeats (SSR) and random amplified polymorphic DNA (RAPD) (Bredemeijer *et al.,* 1998, Villand *et al.,* 1998, Park *et al.,* 2004 and Garcia-Martinez *et al.,* 2006).

The RAPD is the first PCR-based molecular markers technique to develop DNA marker for detecting and monitoring pedigree breeding record of inbred parents or varieties evaluation in test crosses and determining genetic relationships among genotypes (Dongre and Parkhi, 2005). It is an efficient method for varietal identification, study of polymorphism, gene mapping, biodiversity, genetic map construction, hybridization and phylogenetic relationship in tomato varieties (Sharma and Sharma, 1999).

The aim of present study is to observethe morphological characters, genetic diversity within different tomato accessions by using random amplified polymorphic DNA (RAPD) markers and development of phylogentic tree by using bio informatics tools. Moreover, enterprise the protocol for DNA extraction and PCR amplification and development of phylogentic tree of different tomato accessions by using bio informatic tools.

MATERIALS AND METHODS

Seeds of tomato accessions obtained from National institute of Genetic resources (Islamabad, Pakistan) were grown in green house for germination and growth. After two weeks of sowing sprouting started in almost all accessions and after 4-5 weeks of regular watering these plants were grown at a fair height of 60-70 cm.

Table 1. Name of Tomato accessions for DNA extraction

1.	Tm 006233	2.	Tm 017856	3.	Tm 017860	4.	Tm 017869	5.	Tm 017870
6.	Tm 017872	7.	Tm 017873	8.	Tm 017874	9.	Tm 017875	10.	Tm 017876
11.	Tm 017877	12.	Tm 017878	13.	Tm 019842	14.	Tm 019843	15.	Tm 019844
16.	Tm 019846	17.	Tm 019849	18.	Tm 019851	19.	Tm 019852	20.	Tm 019853
21.	Tm 019855	22.	Tm 019856	23.	Tm 019857	24.	Tm 019860	25.	Tm 101159

Morphological Characterization of Tomato accessions: Morphological characters were studied in selected tomato accessions by already set standards for morphological characters by IPGRI (International Plant Genetic Resources Institute) tomato descriptor (Darwin *et al*., 2003). These Characterizations include the plant growth type and size, leaf shape, size and arrangement, plant height and fruit morphology i.enumber of fruits per plant, immature and mature colure. For Molecular characterizationRAPD (Random Amplified Polymorphic DNA) analysis selected for the estimation of genetic diversity and phylogeny among these tomato accessions.

DNA extraction method: Total genomic DNA was isolated from fresh and healthy leaves using the CTAB (hexadecyltrimethylammonium-bromide) method (Murray and Thompson, 1980) with few modifications. Briefly, 1g of leaves was ground in liquid nitrogen to a fine powder. The powder was added to 3 mL of extraction buffer (100 mMTris–HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (wv^{-1}) CTAB, 2–mercaptoethanol 2% and incubated at 65 °C for 30 min. The DNA was extracted with chloroform – octanol (24: 1). The DNA was washed with 70% ethanol and dissolved in 100-400 µl of T.E (10 mMTris–HCl pH 8.0, 1 mM EDTA and 0.2 -1 mg/ml RNAase). DNA is quantified by spectrophotometer by using a comparison of the optical density values of the solution at A_{260}/A_{280} wavelengths. Stock DNA samples were stored at –20 °C and diluted to 20 ng μL^{-1} when it use.

RAPD analysis: The RAPD primers were purchased from Invitrogen product (Invitrogen, USA). A total of 25 decamer oligonucleotides of arbitrary sequence were tested for PCR amplification. The basic protocol reported by William *et al.* (1990) for PCR was performed in a total volume of 15 µl, containing 20 ng μL^{-1} of template DNA, 0.4 µM of single primer, 0.6 U Taq DNA polymerase (Invitrogen, USA) 0.20 µM of dNTPs, 1.5 mM $MgCl_2$, 10 mMTris-HCl,and 50 mMKCl.

DNA amplification was carried out in advanced Primus-96 Thermal cycler and the thermal cycler conditions for PCR reactions were an initial denaturation cycle of 94°C for 4 minutes was followed by 35 cycles comprising 30 sec at 94°C, 1 min at 36°C and 2 min at 72°C. An additional cycle of 7 min at 68°C was used for final extension. Amplification products were separated by electrophoresis in 1.0 % agarose gels and stained in ethidium bromide. A photographic record was taken under UV illumination.

Statistical analysis: Only clear and repeatable amplification products were scored as 1 for present bands and 0 for absent ones. The specific bands useful for identifying different varieties were named with a primer number followed by the approximate size of the amplified fragment in base pairs. Polymorphism was calculated based on the presence or absence of bands. The 0 or 1 data matrix was created and used to calculate the genetic distance and similarity using DNAMANN software. The dendrogram was constructed by using a distance matrix using Nie and Lie's Coefficients to access the genetic similarity and dissimilarity among all accessions.

RESULTS

Morphological characterization: All25 selected tomato accessions were characterized morphologically in this study by comparing the height of plant, leaf length, shape and arrangement, fruit shape and size.

This study revealed that maximum height of plant was 80 cm in Tm 019856 while minimum height of plant was 20 cm in Tm 019843.The maximum leaf length (7.8 cm)was noted in Tm 019856and minimum length(5 cm) was found in Tm 019846.In fruit morphology fruit colour was light green (at immature stage) and red (at mature stage) was observed with rounded in shape. Similarly Maximum fruit size (4.5 cm) was obtained by Tm 019853 while minimum fruit size (2 cm) was found in Tm 017870.

RAPD-PCR characterization of varieties:

Genetic polymorphism amongtomato accessions: After screening 25 primers, 15 primers produced polymorphic and repeatable products. The banding profile and polymorphism generated using the primer (TP-05) (Figure 4.2.3) are shown. PCR amplification of the DNA isolated from 25 selected tomato accessions yielded a total of 130 amplified products out of which 98 were polymorphic and 32 were monomorphic (Table 2). The total no. of DNA bands amplified varied between 05 (Primer TP- 09 andTP-13) and 14 (Primer TP-01) with the average of 8.6 bands per primer. The maximum no. of polymorphic bands (14) was obtained with primers TP-01 and the minimum number (05) was obtained with primer TP-09 and primer TP-13.The polymorphism percentage ranged from 42.85% (Primer TP-07) to as high as 91.66% for primer (TP-05). Average polymorphism across 25 selected tomato accessions was found to be 72.6%. Overall size of the PCR amplified product bands ranged from 400bp to 2500 bp. Polymorphism analysis was done for 25 tomato accessionsOut of these25, 15tomato accessionswere monomorphic and 10 were polymorphic. Primer TP 05 generated maximum polymorphic bands and Primer TP 08 produced the minimum number of bands. The highest level of polymorphism was detected with primer CP 05 (91%) whereas primer TP 08 detected the least polymorphism (42%). (Table 2).

Multiple sequence alignment by DNAMAN software: The multiple alignments of the sequences generated by using 0-1 matrix method for gel scoring. DNAMAN Software aligned the similar sequences and showed a sequence similarity index of 75.60% in the DNA of 25 selected tomato accessions with dissimilarity index of 24.4%. Phylogenetic tree wasgenerated for phylogenetic studies among 25 tomato accessions in the present study.

DISCUSSION

The main aim of present study was to characterize different tomato accessions in Pakistan through morphological and molecular markers and then the generation of phylogenetic tree for these varieties on the basis of RAPD fingerprints. Morphological characterization included the stem and plant growth type, plant height (before transplanting), leaves type, size and arrangement, inflorescence type and exterior colour of immature and mature fruit, shape and size were studied.

Molecular characterization was carried out through RAPD molecular technique by using 25 decamer primers, out of which 15 primers showed polymorphism. Genomic DNA was amplified by 15 polymorphic primers that generated a total 130 loci in all varieties. Maximum number of loci 14 was obtained in the genome of Tm 019851 and minimum number of loci 05 in the genome of Tm 017872.

Polymorphism was estimated between 25 tomato accessions by 25 decamer primers with different sequence out of which 15 primers showed about 72.6% polymorphism in all accessions of tomato. By using 08 decamer RAPD primers, 228 DNA bands were obtained among 36 tomato cultivars (Huh*et al*., 2011). They also found total of number of bands generated by eachprimer was range from 2 to 11 with an average of 6.3 bands per primer. Bernardette *et al.,* (2006) also estimated the variability of 35 tomato accessions in which 257 reproducibly bands were obtained from 20 primers with 78.6% polymorphic. Seventy-four (74) amplified products were scored with 62.2% of polymorphism in 14 tomato varieties were reported byEzekiel*et al*., (2011).

Phylogenetic dendrogramme was constructed among selected varieties by using RAPD fingerprints through DNAMAN computerizes software. Elham *et al*., (2010) also reported the phylogenetic diversity and relationships of some tomato varieties by using RAPD analysis and Ezekiel *et al*., (2011) studied the genetic diversity in 14 tomato varieties by RAPD-PCR technique.

Table 2. Identity, sequence, number of total bands, range of fragment size, polymorphic bands and polymorphic percentage for 10 RAPD decamer polymorphic primers used on 25 selected tomato accessions.

Primer	Primer Sequence	Total number of loci produced	Range of band size(bp)	Total number of polymorphic bands	Rate of Polymorphism %
01	AGTCAGCCAC	14	500 -1400	11	78
02	GTTGCCAGCC	12	400 - 1500	10	83
03	GGGGTGACGA	07	400 - 2500	04	57
04	GAGACGCACA	05	500 -1200	03	60
05	AACGGCGACA	12	400 - 2000	11	91
06	CAGAAGCGGA	06	400 -1000	04	66
07	GACACGGACC	09	500 - 1500	08	88
08	CTCACCGTCC	07	500 - 1300	03	42
09	CCACAGCAGT	07	500 - 1200	05	71
10	ACGACCGACA	11	500 - 1300	09	81
11	AGGGAACGAG	09	500-1400	07	77
12	AGGTGACCGT	06	400-1200	04	66
13	CTGCTGGGAC	06	500-1500	05	83
14	GTGAGGCGTC	10	400-2000	08	80
15	AATCGGGCTG	09	600-1500	06	66
Total	15	130		98	72.6%

Morphological characterization:

Fig.1. (a) Plant height(b) Leaves shape and arrangement(c) Fruit no. colour and size

Fig. 2. (a) Plant height(b) Leaves shape and arrangement(c) Fruit colour and size

Fig.3. (a) Plant heightat stage 2(b) Leaf shape(c) Fruit colour and size Phylogenetic tree

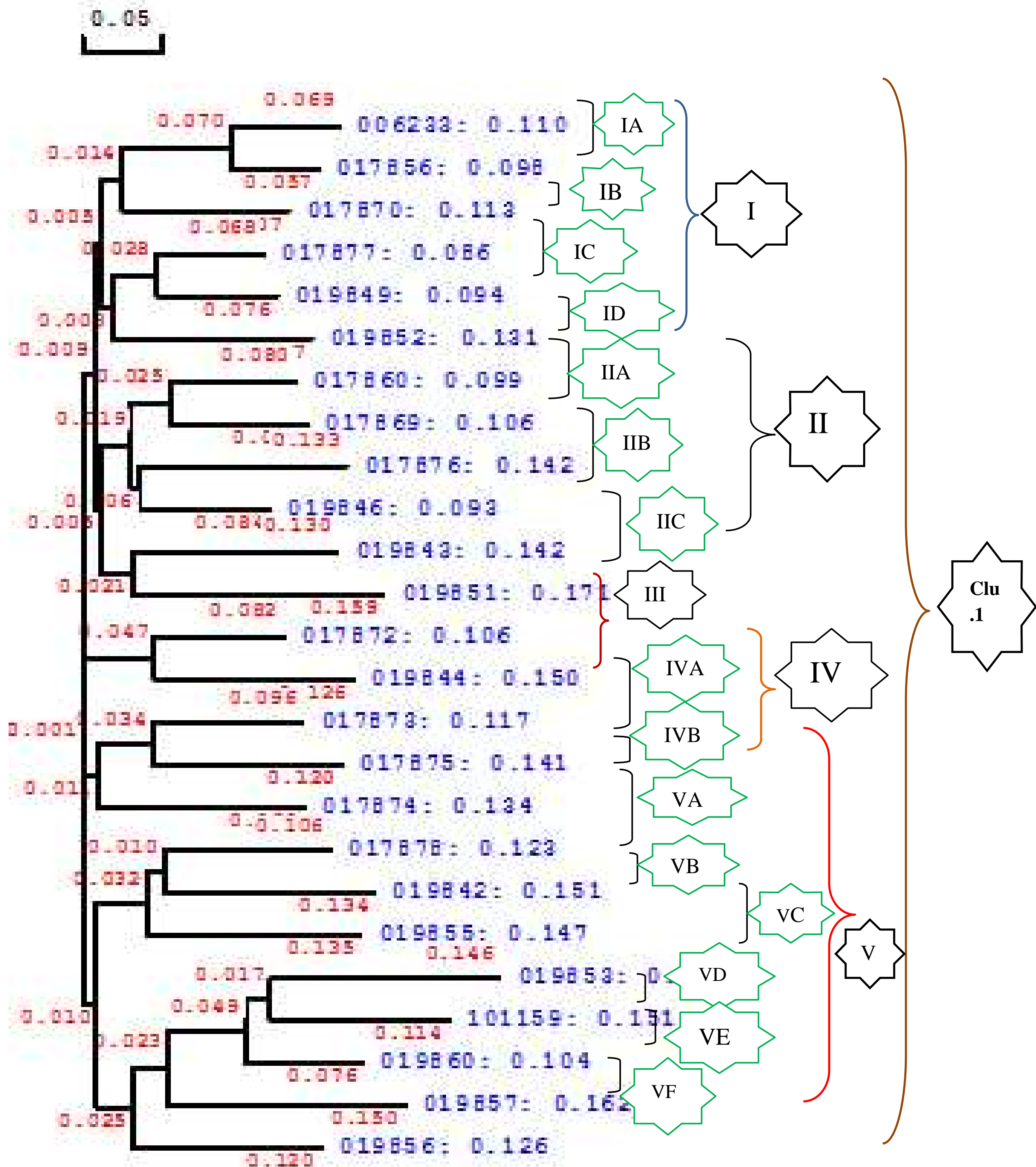

Fig. 4. Phylogenetic tree among 25 tomato accessions by DNAMAN software (LinnonBiosoft, Vaudreuil, Quebec, Canada, version 5.2.2) that was based on Neighbor- joining algorithm of Saitou and Nei (1987). The bar represents a standard distance unit of 0.05 when length of branches was calculated. The number followed by the name of the variety is the sequence weight with numerical values on branches is distance confidence values.

Primer 02.
Ladder 01 02 03 04 05 06 07 08 09 10 11 12 13 14 15 16 17 1819 20 21 22 23 24 25

Figure.4. 2.1:V1,V2,V3,V4,V4, V5, V6,V7,V8, V9,V10, V11,V12,V13, V14, V15,V16,V17, V18,V19, V20,V21, V22,V23, V24,V25

Primer 03.
Ladder 01 02 03 04 05 06 07 08 09 10 11 12 13 14 15 16 17 1819 20 21 22 23 2425

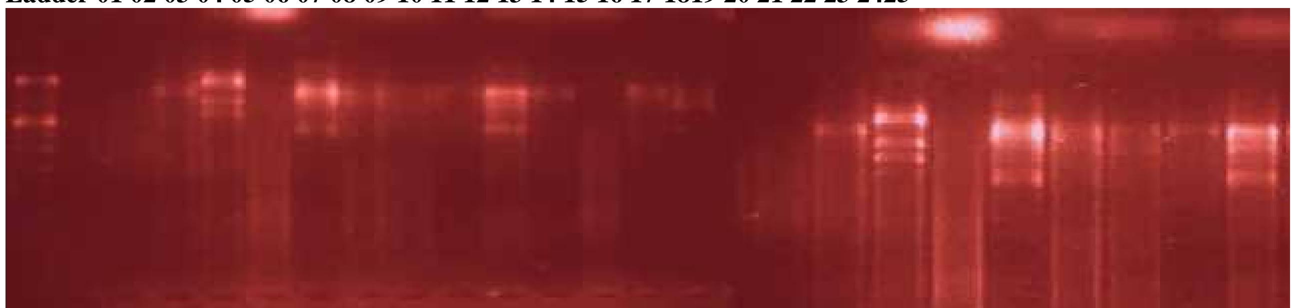

Figure.4. 2.2: V1,V2,V3,V4,V4,V5,V6,V7,V8,V9,V10, V11,V12,V13, V14, V15,V16, V17, V18,V19, V20, V21,V22,V23, V24,V25

Primer 05.
Ladder 0102 03 04 05 06 07 08 09 10 11 12 13 14 15 16 17 1819 20 21 22 23 24 25

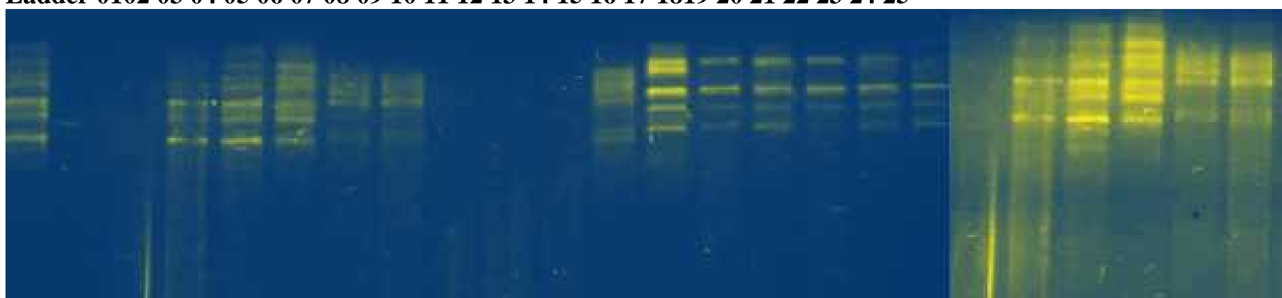

Figure.4. 2.3:V1, V2,V3,V4,V4, V5, V6,V7,V8, V9,V10, V11,V12,V13, V14,V15,V16,V17, V18,V19,V20,V21, V22,V23, V24,V25

Primer 07:

Ladder 01 02 03 04 05 06 07 08 09 10 11 12 13 14 15 16 17 1819 20 21 22 23 24 25

Figure.4. 2.4: V1, V2,V3,V4,V4, V5, V6,V7,V8, V9, V10, V11,V12,V13, V14, V15,V16,V17, V18,V19, V20, V21, V22,V23, V24,V25

Primer 09:
Ladder 01 02 03 04 05 06 07 08 09 10 11 12 13 14 15 16 17 1819 20 21 22 23 2425

Figure.4. 2.5: V1, V2, V3,V4,V4, V5,V6,V7,V8, V9, V10,V11,V12,V13, V14, V15,V16,V17, V18,V19, V20, V21, V22,V23, V24,V25

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