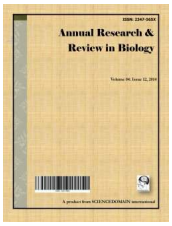

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# Genome Wide Identification and Analysis of Microsatellite Repeats in the Largest DNA Viruses (*Poxviridae* Family): An *Insilico* Approach

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors KKB, SA and KKM designed the study, performed identification of microsatellites, wrote the protocol and wrote the first draft of the manuscript. Author MRGB performed evolutionary studies of poxviruses and managed the manuscript. Authors RY and GMR performed the statistical analysis and managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Background:** Microsatellites also known as simple sequence repeats (SSRs), which is also called as junk DNA, mainly used as a neutral genetic marker, presented across coding and non-coding regions of prokaryotes, eukaryotes, and viruses. They are subjects of different fields, such as gene mapping, population genetics, DNA fingerprinting, forensic studies and evolution.

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**Aim:** The presented study is focused on the evolutionary relationship between poxviruses for the identification and systematic analysis of the nature and distribution of complex microsatellites, presenting in large DNA viral genomes of poxviruses (Poxviridae family) in vertebrates and invertebrates.

**Materials and Methods:** Genome sequences of seventeen species from the Poxviridae family were assessed by the National Center for Biotechnology (NCBI). The microsatellite was extracted using IMEx software, and statistical analysis was performed using Microsoft office Excel 2007. Furthermore, the molecular evolutionary analyses of poxviruses were conducted using MEGA6.

**Results:** In the current study, we screened 17 vertebrate and invertebrates of pox viral genomes and a total of 8539 SSRs which revealed a total of 2387 cSSRs distributed across all the genomes. From the sequences, poly A or poly T mononucleotide prevailed over a poly G or poly C. Among the identified motifs dinucleotides 51.73% which were the most common types of repeats followed by mononucleotides 36.12%, trinucleotides 11.28%, tetranucleotides 0.56%, pentanucleotides 0.10%, and hexanucleotides 0.21%. Polymorphism increases with genome length and decreasing GC content of repeat motifs for dinucleotides, trinucleotides, and tetranucleotides. This result may help genome-wide evolutionary and quantitative analysis like genome size or GC content which has an influence on the number, simple and compound microsatellite of relative abundance and relative densities.

**Conclusion:** We conclude that largest DNA virus of invertebrates show a higher percentage of microsatellites and repeat motif than the vertebrate poxviruses. The genome size and GC content is an important factor in affecting the occurrence of repeat motif as well microsatellites, in vertebrate and invertebrate poxviruses. The analysis on the phylogenetic relationships and microsatellites in vertebrates and invertebrates, as well the pattern of their evolution, may help to understand (the understanding) of poxviruses in the course of natural evolution.

*Keywords: Poxviridae; SSR; cSSR; relative density; relative abundance; compound.*

## 1. INTRODUCTION

The poxviruses belong to the family *Poxviridae*. These pathogenic viruses cause disease which infects both humans and animals (wild and domestic) [1,2]. The poxviruses are enveloped, brick-shaped virion, large and complex viruses with a size of approximately 220 - 450 nm long and 140-260 nm wide [3]. The genome of poxviruses is linear dsDNA, containing 130-360 kilobase pair with a hairpin loop at each end [4,5] encoding more than 150 genes per genome. The replication of poxvirus is complex and it occurs in the cytoplasm [6,7] preventing the virus from using nuclear enzymes of the host; encoding its own enzymes for DNA replication, but the cellular functions are necessary for viral maturation [8]. Comparative DNA sequencing studies have found that these genes are located in the poxvirus genome, which tend to be conserved highly [9] and share the similar genetic organization with the genes involved in all important functions such as transcription, replication and virion assembly [10]. The morphogenesis of the virion is of two classes i.e., intracellular mature virions (IMV) and extracellular enveloped virions (EEV) which initiate the infectious cycle and produce acute viral diseases [11,12]. The genes show more variability in terms of sequence homology and are involved in immunomodulation and host range determination among poxvirus family members [13,14].

The *Poxviridae* family is divided into two subfamilies such as *Entomopoxvirinae* and *Chordopoxvirinae* which includes Poxviruses of insects and poxviruses of vertebrates. The Chordopoxviruses subfamily is further classified into different genera, including Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus, Cervidylipoxviruses, Crocodylipoxviruses and Yatapoxvirus [15,16]. These groups of Poxviruses are pathogenic and affect livestock, animals, and humans [3,17]. Orthopoxviruses, Parapoxviruses, Yatapoxviruses, and Molluscipoxviruses are members of the Chordopoxvirinae which infect humans [4,18]. Entamopoxvirinae, the subfamily of *poxviridae* family infects the larvae of insects [19,20]. Alphaentomopoxvirus and Gammaentomopoxvirus genomes contribute to better discernment and relationships among the Entamopoxviruses and Chordopoxviruses [21]. EPVs have been studied primarily because they

are potential insect biocontrol agents and also used as expression vectors [22] these recombinant poxviruses may become useful for the vaccination [23]. SSRs or junk DNA are used as genetic and molecular markers [24,25]. While recent surveys have documented that they could play crucial roles in affecting gene activity, chromatin organization and DNA metabolic processes [26]. These repeats have been extensively used in the molecular biology, genetic mapping, phylogenetic mapping [27], paternity analysis and population studies [28].

Microsatellites are ubiquitously distributed in many prokaryotes, eukaryotes and viral genomes [29,30,31] and they provide a molecular basis for quick adjustment to environmental changes [26]. Some pathogens are found to have the ability to utilize SSRs to frustrate the host immune system by using it to enhance their antigenic variability [32]. Characterization of the genome variability in these viral populations is necessary because those data could provide useful information on the process and the regulation of the virus' evolution [33]. Identification and analysis of these SSRs in diverse vertebrate and invertebrate poxviral genomes would help in emerging the course of natural growth and comparative analysis of these repeat sequences. Therefore we studied the natural case, size, density and distribution of different microsatellites in diverse species of vertebrates and invertebrate poxviral genomes, which can aid in understanding the origin and evolution of repeat sequences, genome evolution and adaptation to divergent hosts.

## 2. MATERIALS AND METHODS

### 2.1 Analysis of Poxviruses Genomes

The poxviral genome sequences were assessed from NCBI and analyzed in FASTA format. Phylogenetic relationship among all poxviruses was constructed using the Mega 6 software packages [34]. The nucleotide size ranges from nt 288539 (Acc No - AF198100) to 139962nt (Acc No - NC_005336). The accession numbers and salient features of *poxviridae* family genomes have been summed up in Table 1.

### 2.2 Microsatellite Identification and Analysis

The microsatellite identification was performed using the IMEx software [35]. Advanced-mode of IMEx was used to identify simple and compound microsatellite repeats; the parameters used here was described earlier for the microsatellite analysis of Ebola virus and for the analysis of the largest RNA virus family [30,33]. The parameters used are Types of repeat: perfect; repeat size: all minimum repeat numbers: 6, 3, 3, 3, 3, 3; maximum distance allowed between any two SSRs (DMAX): 10 [36].

### 2.3 Statistical Analysis

The statistical analysis was performed by Microsoft Office Excel packages 2007. Linear regression was used to detect the correlation between the relative abundance and relative density of microsatellites with genome size.

**Table 1. An overview of the poxviral genomes used for the study**

| Sl. no | Species Id | Name | Acc no | Genome size (bp) | GC% |
|---|---|---|---|---|---|
| 1 | P1 | Camelpox virus | AY009089 | 202205 | 33.2 |
| 2 | P2 | Cowpox virus | AF482758 | 224499 | 33.4 |
| 3 | P3 | Deerpox virus | AY689436 | 166259 | 26.16 |
| 4 | P4 | Fowlpox virus | AF198100 | 288539 | 30.89 |
| 5 | P5 | Goatpox virus | AY077835 | 149599 | 25.31 |
| 6 | P6 | Horse pox | DQ792504 | 212633 | 33.09 |
| 7 | P7 | Lumpy skin disease virus | AF325528 | 150773 | 25.91 |
| 8 | P8 | Molluscum contagiosum virus | U60315 | 190289 | 63.36 |
| 9 | P9 | Monkeypox virus strain | AF380138 | 196858 | 33.09 |
| 10 | P10 | Nile crocodilepox virus | DQ356948 | 190054 | 61.92 |
| 11 | P11 | Orf | NC_005336 | 139962 | 63.44 |
| 12 | P12 | Sheeppox virus | AY077832 | 149955 | 25.01 |
| 13 | P13 | Swinepox virus | AF410153 | 146454 | 27.4 |
| 14 | P14 | Vaccinia virus | M35027 | 191737 | 33.4 |
| 15 | P15 | Variola virus | X69198 | 185578 | 32.73 |
| 16 | P16 | Entomopoxvirus (Alphaentomopoxvirus) | NC_023426.1 | 245717 | 19.98 |
| 17 | P17 | Adoxophyes honmai entomopoxvirus 'L'(Betaentomopoxvirus) | NC_021247 | 228750 | 21 |

## 3. RESULTS

### 3.1 Phylogenetic Analysis

Seventeen nucleotide sequences retrieved from NCBI (Table 1) were analyzed. All positions containing gaps and missing data were wiped out. There were a total of 103903 positions in the final dataset. The phylogenetic trees of pox viral genomes are showing the isolates that are clustered with different genes (Fig. 1). All the genome sequences are forming the closest neighbouring clusters. Camel pox viral sequences forming a clade have the evolutionary relationship of same genomes with the highest bootstrap value of 99, indicating that it has a uniform support. The dependability of a branch length in MEGA 6 is based on confidence probability (CP). The branch length is high when the confidence probability is high so that the branch length is considered to be statistically significant. The bootstrap value of 1000 with the constructed sequences indicate that the clade is close to 100%, which reveals that all the characters in this group believed to comprise all the evolutionary descendants of a common ancestor which is rooted with different viral genome as the ancestral group [37].

### 3.2 Analysis of Simple and Compound Microsatellites

Genome-wide scan of poxviral genomes with IMEx showing a total of 8539 SSRs which is distributed across all the species with varying incident frequencies ranging from 3380 in P17 (Acc No - NC_021247) to 1171 in P10 (Acc No - DQ356948) (Table 2, Fig. 2a). Further, the relative abundance values lie between a lower limit of 6.16 for P10 to an uttermost of 14.78 bp/kb for P17 (Table 2, Fig. 3a). Comparatively, relative density of SSRs varies from 42.25 bp/kb in P15 to 102.69 bp/kb for P17 (Table 2, Fig. 4a).

In case of cSSRs, IMEx scan showing a total of 2387 cSSRs (Table 2, Fig. 2b). Ubiquitous presence of cSSRs, ranging from 86 in P15 (Acc No- X69198) to 502 in P17. Further, the relative abundance values lie between a lower limit of 0.46 in P14 to a uttermost of 2.19 bp/kb in case of P17 (Table 2, Fig. 3b). Comparatively, the relative density of cSSRs varies from 8.74 bp/kb in P12 to 44.35 bp/kb in P17 (Table 2, Fig. 4b).

### 3.3 Diversity of Extracted SSR Motifs

Mononucleotide repeats were observed in all the poxviral genomes. Poly (A/T) repeats being more prevalent than poly G/C repeats. A maximum of 612 ‘A’ repeats was observed in genome of Adoxophyes honmai entomopoxvirus 'L'(Betaentomopox virus) (P16) in the insect poxviral genome and an upper limit number of 598 poly ‘A’ poxviral genome in the sheep pox virus. Further, a maximum number of 1704 dinucleotide repeats were also observed in

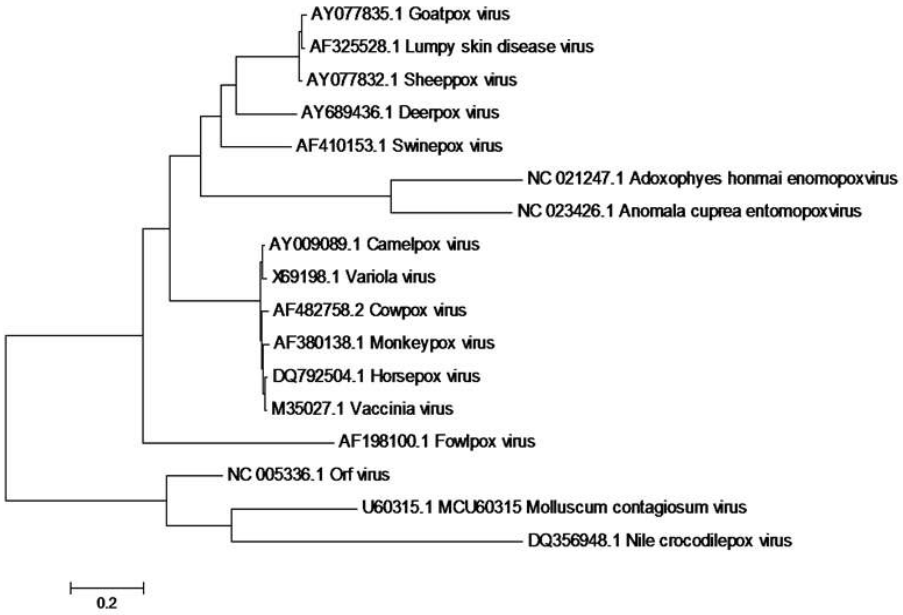


**Fig. 1. Molecular phylogenetic analysis by maximum likelihood method**

*Anomala cuprea* entomopoxvirus (Alphaentomopox virus) invertebrates and 1430 dinucleotide repeats in Molluscum contagiosum virus in vertebrates. Three nucleotide repeats were observed in Nile crocodile pox virus (267) and 484 in *Anomala cuprea* entomopoxvirus (Alphaentomopox virus). Poxviral genomes have 9.7 % of tetranucleotide repeats. The occurrence of average SSR motifs among the poxviral genome is shown in Fig. 5.

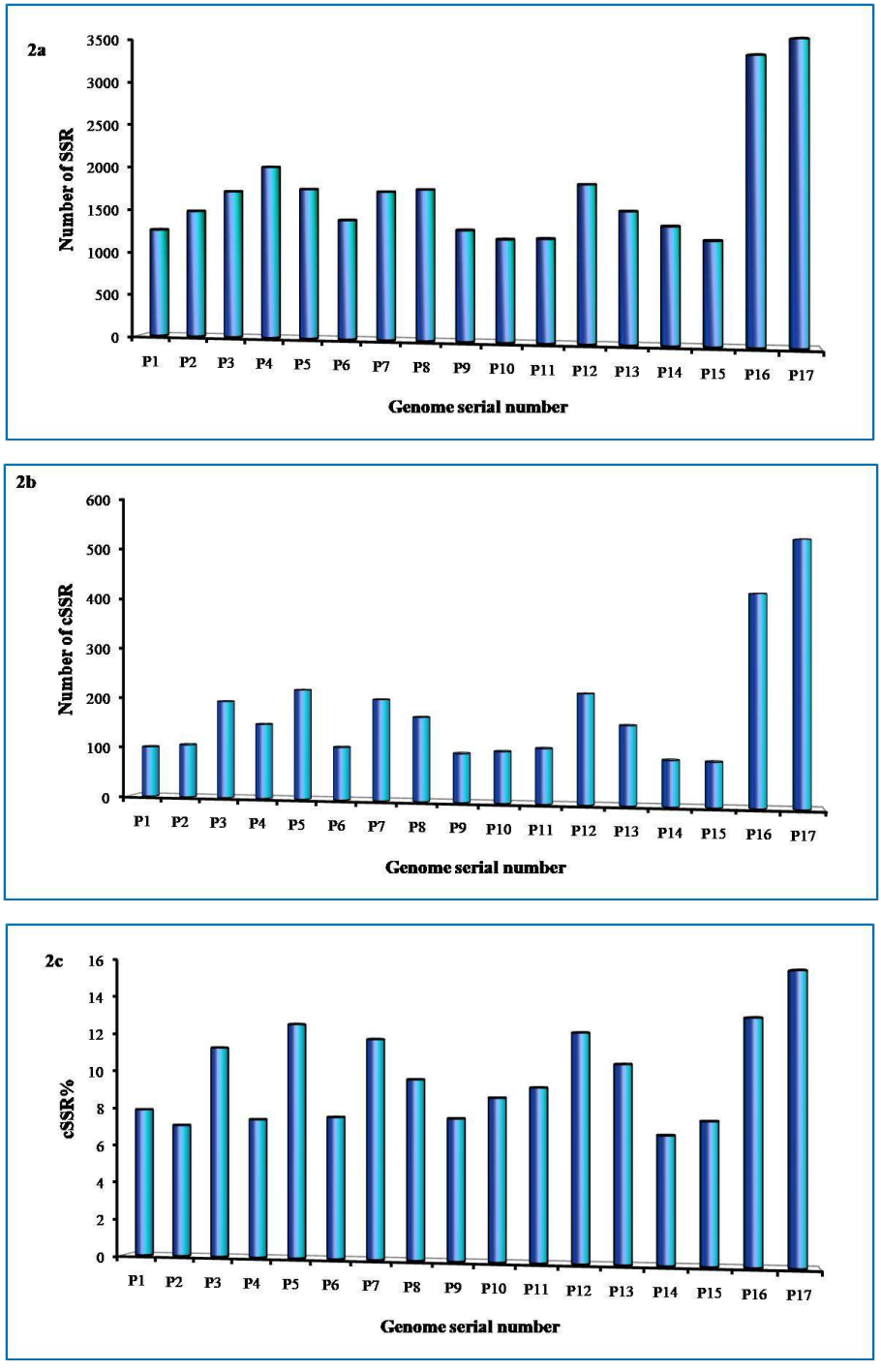


**Fig. 2. Analysis of Simple Sequence Repeats. 2a) Distribution of SSRs; 2b) Distribution of cSSRs; 2c) cSSR% across the *Poxviridae* family**

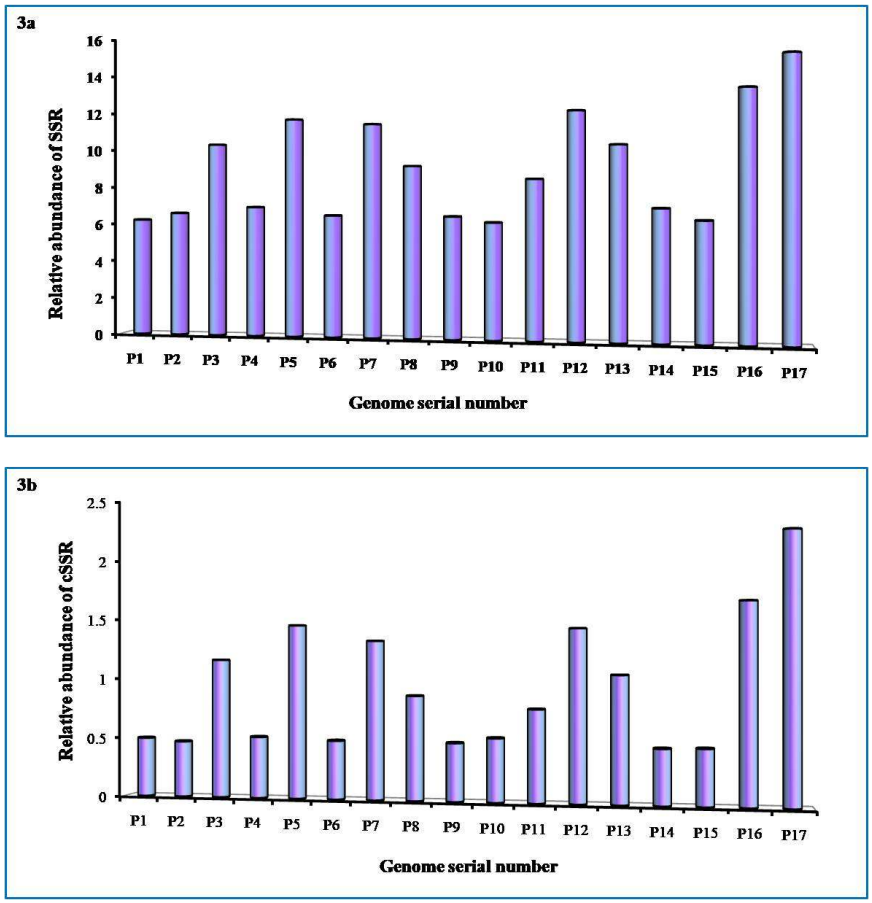


**Fig. 3. Relative Abundance: Simple Sequence Repeats 3a) Compound simple sequence repeats 3b) Present per kilo base of genome**

**Table 2. Overview of various microsatellites, Relative abundance and density of various simple repeat sequences detected in selected poxviral genomes**

| Sl. no | Species Id | SSR | RA | RD | cSSR | cRA | cRD | cSSR% |
|---|---|---|---|---|---|---|---|---|
| 1 | P1 | 1260 | 6.23 | 42.4 | 100 | 0.49 | 9.476 | 7.937 |
| 2 | P2 | 1482 | 6.6 | 45.13 | 105 | 0.47 | 9.314 | 7.085 |
| 3 | P3 | 1714 | 10.3 | 68.99 | 193 | 1.16 | 21.77 | 11.26 |
| 4 | P4 | 2000 | 6.93 | 45.73 | 148 | 0.51 | 9.486 | 7.4 |
| 5 | P5 | 1740 | 11.6 | 79.91 | 217 | 1.45 | 29.8 | 12.47 |
| 6 | P6 | 1381 | 6.49 | 43.72 | 104 | 0.49 | 9.284 | 7.531 |
| 7 | P7 | 1711 | 11.3 | 77.49 | 199 | 1.32 | 27.39 | 11.63 |
| 8 | P8 | 1736 | 9.12 | 67.05 | 165 | 0.87 | 17.98 | 9.505 |
| 9 | P9 | 1273 | 6.47 | 43.97 | 95 | 0.48 | 10.12 | 7.463 |
| 10 | P10 | 1171 | 6.16 | 44.96 | 100 | 0.53 | 10.88 | 8.54 |
| 11 | P11 | 1181 | 8.44 | 59.51 | 107 | 0.76 | 17.37 | 9.06 |
| 12 | P12 | 1795 | 12 | 81.94 | 213 | 1.42 | 28.63 | 11.87 |
| 13 | P13 | 1494 | 10.2 | 69.29 | 153 | 1.04 | 21.09 | 10.24 |
| 14 | P14 | 1329 | 6.93 | 46.13 | 88 | 0.46 | 8.736 | 6.622 |
| 15 | P15 | 1172 | 6.32 | 42.25 | 86 | 0.46 | 8.8 | 7.338 |
| 16 | P16 | 3210 | 13.1 | 91.33 | 402 | 1.64 | 33.99 | 12.52 |
| 17 | P17 | 3380 | 14.8 | 102.7 | 502 | 2.19 | 44.35 | 14.85 |

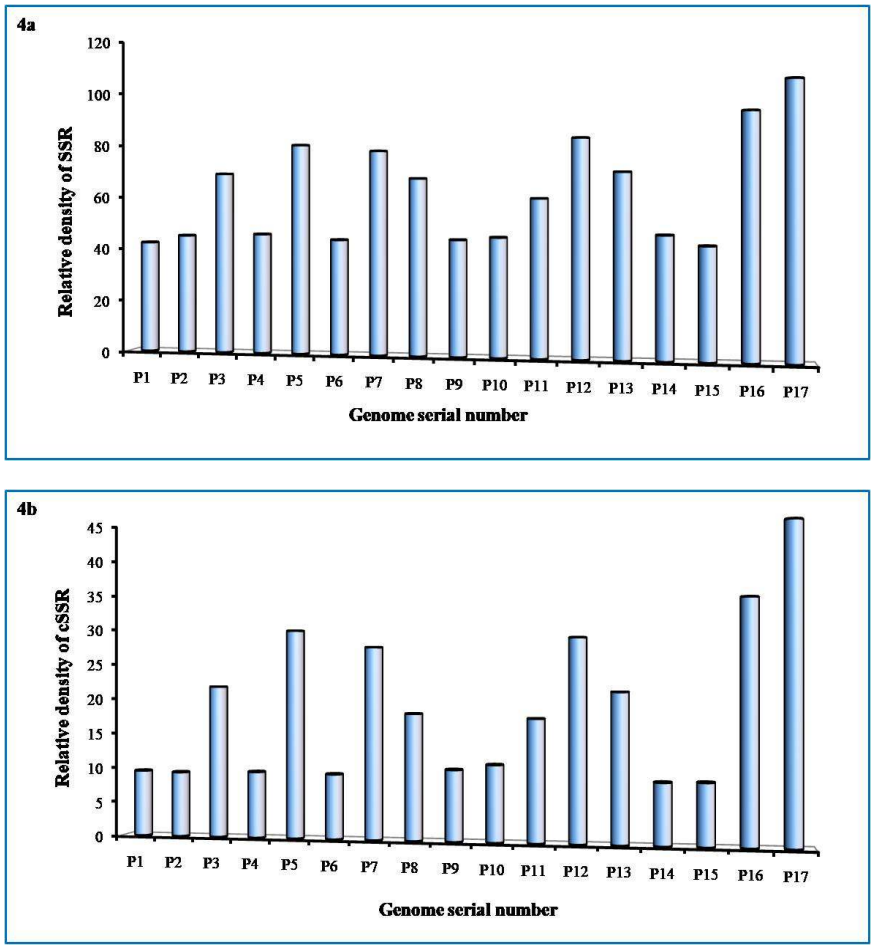


**Fig. 4. Relative density: Total length covered by Simple Sequence Repeats 4a) Compound simple sequence repeats 4b) Per kilo base of genome**

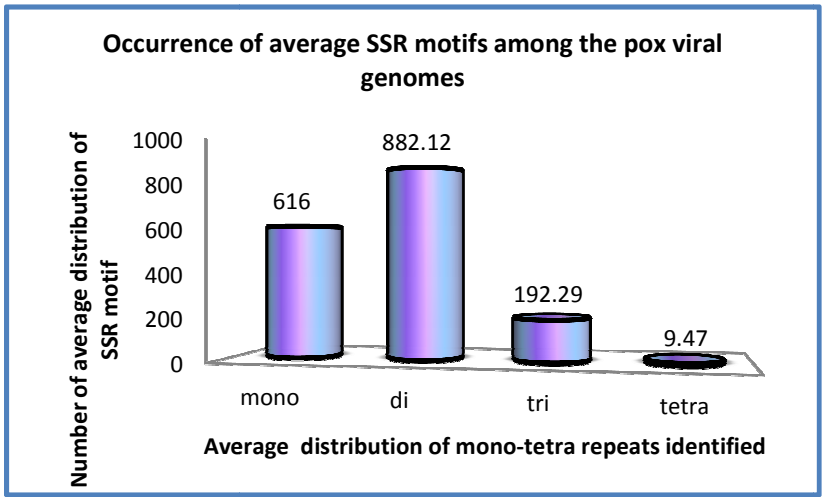


**Fig. 5. Occurrence of average SSR motifs among the pox viral genomes**

### 3.4 Genome Parameters and SSR/cSSR Distribution

Karlpearsons co-efficient of correlation was tested between Genome size (bp), GC%, RA, RD, cSSR, cRA, cRD, cSSR% of SSRs and cSSRs. Incidence of SSRs was non-significantly correlated ($R^2$ = 0.0954, *P* = 0.05) with Genome size (bp) and GC% content ($R^2$ = 0.1186, *P* = 0.05) similarly RA ($R^2$ = 0.4146, *P* = 0.05), RD ($R^2$ = 0.4107, *P* = 0.05), cSSR ($R^2$ = 0.0962, *P* = 0.05), cRA ($R^2$ = 0.3575, *P* = 0.05), cRD ($R^2$ = 0.3582, *P* = 0.05), cSSR% ($R^2$ = 0.2599, *P* = 0.05) of SSRs. The regression analysis of cSSR, non-significantly correlated ($R^2$ = 0.0031, *P* = 0.05) with Genome size (bp) and GC% content ($R^2$ = 0.1325, *P* = 0.05) similarly RA ($R^2$ = 0.3274, *P* = 0.05), RD ($R^2$ = 0.8669, *P* = 0.05), cSSR ($R^2$ = 0.5905, *P* = 0.05), cRA ($R^2$ = 0.8321, *P* = 0.05), cRD ($R^2$ = 0.8669, *P* = 0.05), cSSR% ($R^2$ = 0.8425, *P* = 0.05) of cSSRs. It is clear from the data that SSR depend upon cSSR. Karlpearsons co-efficient of correlation was also determined between the Genome size (bp) and SSR, GC%, RA, RD, cSSR, cRA, cRD, cSSR% which was 0.5443, 0.7201, 0.0184, 0.0182, 0.0776, 0.0069, 0.1743, 0.0315 respectively ($R^2$, *P* = 0.05). The results obtained are co-insiding with the results of Chordopoxviruses which are showing the correlation between genome size with RA and RD equal to 0.0929 and 0.0435 respectively ($R^2$, *P* = 0.05) [10].

## 4. DISCUSSION

In the current study, SSRs were identified and characterized among poxviral genomes. The occurrences of SSRs mononucleotide to hexanucleotide were proportional to the genome size of the *Poxviridae* family. The obtained results revealed totally 8539 SSRs, distributed across all the pox viral genomes. SSR motifs were observed in all genome sequences and the identified SSR motifs are dinucleotides (14996 - motifs; 51.73%) which were the most common types of repeats followed by mononucleotides (10472 - 36.12%), trinucleotides (3269 - motifs; 11.28%), tetranucleotides (161 - motifs; 0.56%), pentanucleotides (30 - motifs; 0.10%), and hexanucleotides (62-0.21%) motifs. More or less of the poxviral genomes the pentanucleotide motif repeats (Camelpox virus, Horse pox, Nile crocodile pox viruses, Orf, vaccine virus) and hexa nucleotide motif repeats (Deerpox virus) were absent and was not detected. Approximately 2387 cSSRs revealed as compound microsatellites and are reportedly involved in regulation of gene expression at the functional level of proteins in several species. cSSR incidence decreases with increases in complexity. The cSSR percentage varies between 6.62 - 14.85% in the *Poxviridae* family genomes. A genome-wide study of the microsatellite distribution of dsDNA viruses showing that dinucleotides repeats were the most dominant followed by mononucleotide, trinucleotide, pentanucleotide, tetranucleotide and hexanucleotide repeats. Simple sequence repeats are considered as hot spots for recombination and the dinucleotide motifs are preferred sites for recombination enzymes. Some SSR sequences such as GT, CA, CT, GA, and others may influence the recombination directly through their effects on DNA structure [38]. Trinucleotides repeats are associated with the development of some diseases and important functions [32].

Mononucleotide repeats were observed in all the poxviral genomes with poly (A/T) repeats which are more prevalent than poly G/C repeats. In yeast and E. coli, mononucleotide repeats strongly affect protein expression by virtue of higher error rates of transcription and translation [32]. The sequence composition of repeats determines the abundance of microsatellites.

In Filoviridae family mononucleotide A/T is most prevalent, followed by dinucleotides AC/CA and trinucleotides AAC/CAA. The highest incidence of SSRs (mono-/di-nucleotide motif) was found in the RNA Dependent RNA Polymerase (RDRP) gene, whereas tri-nucleotide motif was maximally localized in nucleoproteins (NP) [39]. The repeats of *Poxviridae* family, AC/CA predominated GC/CG repeats. Dinucleotide repeats are more common than trinucleotide repeats due to an instability of dinucleotide repeats because of the higher slippage rate. These repeat sequences may provide a molecular device for faster adoption to environmental stress, this may accelerate the growth of the *Poxviridae* family.

Some of the microsatellites significantly play important role in poxviral genome organization and evolution. Microsatellites are shown hypermutable regions in viruses [32]. Slippage can destabilize microsatellites because of alterations in DNA polymerase or its cofactors that result in increased slippage rates. In E.coli, microsatellite instability increases due to mutations in the genes of the DNA repair system

which substantially increase (up to 700 times) [24]. These microsatellites are widely used in a diversity of basic principle and applied fields of life and medical skills since they show a high level of polymorphism, relatively small size and rapid detection protocols [40].

## 5. CONCLUSION

This genome-wide study of largest DNA invertebrate poxviruses shows a higher percentage of microsatellites and repeat motif than the vertebrate poxviruses. Genome size and GC contents are the significant factors in affecting the occurrence and the total length of simple sequence repeats in large DNA poxviral genomes, there is a positive correlation between the additional hosts and are correlated to the variety of SSRs content certain degree. Similar genome size, viruses infecting vertebrates and invertebrates tend to be higher than viruses attacking bacteria in SSRs content [41]. Since Microsatellites are short simple sequence repeats, it may accumulate more mutability and often show more variability than surrounding sequences [10]. We concluded that genome size is a significant factor in affecting the occurrence of SSRs; hosts are also responsible for the variances of SSRs content to a certain stage. Insilico mining and compilation of simple sequence repeats (SSRs) in viruses and its analysis with reference to incidence, distribution, and variation would be instrumental in translating the functional and evolutionary aspects of repeat sequences. Taking in consideration the parasitic characteristics of the viruses, studies on the SSRs in poxviruses could be helpful in many research fields, like etiopathogenesis of the respective hosts.

## COMPETING INTERESTS

The authors confirm that there are no conflicts of interest associated with this study and there has been no significant financial support for this work that could have influenced its outcome.

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