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PHYLOGENETIC ANALYSIS OF ANTERIOR-POSTERIOR PATTERNING PROTEINS IN DIFFERENT SPECIES OF *Drosophila* (Diptera: Drosophilidae)

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AUTHORS' CONTRIBUTIONS
This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Drosophila melanogaster has always been the favourite organism for studying insect biology. Most of our understanding regarding insect development comes from this organism. Previous studies have revealed the role of many different proteins in the axes formation of the *Drosophila* embryo. The role of Hunchback, Kruppel, Giant, Knirps, Caudal, Hairy, Even-skipped, Odd Skipped, Fushi-tarazu, Wingless, and Engrailed and other proteins have already been established in the development of *D. melanogaster*. While most of the research work has been carried out on *D. melanogaster*, the other species belonging to the genus *Drosophila* have gotten a little attention. It would be interesting to understand how the proteins involved in insect development evolved in different species of this genus. Therefore, the present study was carried out to analyse phylogenetic relationships and sequence variation among seven different *Drosophila* species using sequences of thirteen different proteins involved in anterior-posterior patterning in the *Drosophila* embryo. The MEGA XI software was used for the phylogenetic analysis which revealed that *Drosophila melanogaster* was the most recently diverged species as far as the A-P patterning proteins are concerned. Maximum variable sites were observed in Hunchback and the minimum in Wingless.

Keywords: Anterior-Posterior patterning proteins; *Drosophila;* phylogeny; MEGA X; variable sites.

1. INTRODUCTION

The study of breeds, using molecular techniques is very important and useful for their characterizing [1,2]. Conservation of diversity in animal species requires the proper performance of conservation superiorities and sustainable handling plans that should be based on universal information on population structures, including diversity resources among and between breeds [3,4]. Diversity is an

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essential element for genetic improvement, preserving populations, evolution and adapting to variable environmental situations [5,6]. On the other hands, determination of polymorphism is important in animals breeding [7,8] in order to define genotypes of animals and their associations with productive, reproductive and economic traits [9-11]. *Drosophila* has been a good model organism for genetics, physiology, development, evolution, and population genetics. Segmentation in *Drosophila* is determined by different sets of gene expression which divide its body into different units. Anterior-posterior fate in *Drosophila* is initially determined by time and morphogen gradients [12]. The boundaries of zygotic genes expression in *Drosophila* are initially established by the maternal gradients. The maternal Bicoid gradient determines the boundaries for *Kruppel* (Kr), *Giant* (gt), *Knirps* (kni) and *Hunchback* (hb) [13,14]. Bicoid forms an anterior to posterior gradient [13]. Hunchback forms a gradient across an anterior-posterior axis, whereas in the posterior region it is blocked by *Nanos* [15]. In the posterior region, *Caudal* activity is mediated through the *Cad* mRNA as its mRNA is ubiquitously present throughout the oocyte, but its activity in the anterior region is blocked by its interaction with *Bicoid* through *BCD cad* 3' UTR forming a posterior to anterior gradient [12]. Therefore, the anterior-posterior patterning in *Drosophila* is controlled by the cascade of different classes of genes. At the top, maternal genes are present, which regulate the expression of Gap genes. Maternal genes with gap genes regulate the expression of pair-rule, which in turn regulates the expression of segment polarity genes [16]. The expression of the patterning gene determines the fate of the cell. The conservation of gene expression helps in maintaining the developmental plan over a lineage, regardless of the change in DNA sequences that encode them. The wealth of information about the different DNA and protein sequences is present in the multiple genomes. Proteins play a significant role in the developmental pathways. A change in protein sequences can influence the expression of a gene. . Moreover, the epigenome comprising different mechanisms e.g. DNA methylation, remodeling, histone tail modifications, chromatin microRNAs, and long non-coding RNAs, interact with environmental factors like nutrition, pathogens, climate to influence the expression profile of genes and the emergence of specific phenotypes [17,18]. Multi-level interactions between the genome, epigenome and environmental factors might occur [19]. Furthermore, numerous lines of evidence suggest the influence of epigenome variation on health and production [17, 20-21]. The expression of eukaryotic genes is temporarily and multidimensionally controlled [22]. Only a relatively small set of the entire genome is expressed in each type of tissue, and the expression of genes depends on the stage of development [23]. Therefore, gene expression in eukaryotes is specific to each tissue [24]. Also, the amount of gene products that are made in the same tissue as well as in other tissues that make up that product, regulates the expression of that gene. One of the basic activities in domestic animals is the study of genes and proteins related to economic traits and their study at the cellular or chromosomal level [25]. So, the current research work was done to study the phylogenetic relationships of the proteins responsible for anterior-posterior patterning among seven different species of *Drosophila* i.e. *Drosophila melanogaster, Drosophila yakuba, Drosophila virilis, Drosophila sechellia, Drosophila arizonae, Drosophila erecta,* and *Drosophila persimilis.*

2. MATERIAL AND METHODS

2.1 Data Collection

The protein sequences responsible for anterior-posterior axis formation belonging to different *Drosophila* species were downloaded from the NCBI (National Center for Biotechnology Information). The accession numbers of the protein sequences used are mentioned in Table 1. The FASTA sequences were used for the BLAST query to find the level of conservation of each protein in different *Drosophila* species. These sequences were then saved in a Microsoft word document.

2.2 Sequence Alignment

The protein sequence saved in the Microsoft word document is aligned with the program **ClustalW** [26] in **MEGA X** [27] software.

2.3 Phylogenetic Tree

The multiple sequence alignments were then used for the construction of a Maximum Likelihood (ML) and Maximum Parsimony (MP) in **MEGA XI** [28] software. To estimate the reliability of the phylogenetic tree bootstrap method was used, with a bootstrap value of 1000.

2.4 Sequence Variation

The number of variable sites and parsimony-informative sites present in the protein sequence were calculated with **MEGA X** [27] software.

Table 1. Table showing the accession number of protein sequences used for phylogenetic analysis

Species name/ Name of genes	***Drosophila melanogaster***	***Drosophila yakuba***	***Drosophila virilis***	***Drosophila sechellia***	***Drosophila arizonae***	***Drosophila erecta***	***Drosophila persimilis***
Hunchback	NP_731268.1	CAA0656.1	KRF8.730.11	CAA06504.1	XP_017874974.1	XP_015010040.1	XP_026844385.1
Caudal	NP_001260641.1	XP_002091242.2	XP_002052691.2	XP_002042467.1	XP_017857978.1	XP_001974115.2	XP_0020152371
kruppel	NP_001261181.1	XP_002092946.1	XP_002048954.1	XP_002043182.1	XP_017868742.1	XP_001976730.1	XP_002018332.2
knirps	NP_001287130.1	XP_002095428.1	XP_002048095.2	XP_002040702.1	XP_017860956.1	XP_001973525.3	XP_026849332.1
Giant	NP_525049.1	XP_002100261.1	XP_002057392.2	XP_002040516.1	XP_017869537.1	XP_001982655.1	XP_002021837.1
Hairy	NP_523977.2	XP_002093420.1	XP_002046765.1	XP_002029764.1	XP_017864240.1	XP_001971618.1	XP_026848773.1
Runt	NP_523424.2	XP_002101924.1	AAA91784.1	XP_002039570.1	XP_017872595.1	XP_001978629.1	XP_002024533.2
Even skipped	NP_523670.2	XP_002089876.2	EDW60048.1	XP_002033172.1	XP_017868748.1	EDV58175.1	XP_002018431.1
Odd-skipped	NP_722922.1	XP_002087851.1	XP_002052531.1	XP_002037738.1	XP_017857317.1	XP_001968586.1	XP_026844369.1
Paired	NP_523556.1	XP_002088442.1	XP_00205203.1	XP_002042021.1	XP_017860538.1	XP_001969768.1	XP_026844785.1
Fushi-tatazu	NP_477498.1	XP_002096728.1	AAO01076.1	XP_002038618.1	XP_017856046.1	XP_001979125.1	XP_002016958.1
Wingless	NP_523502.1	x	XP_002052574.1	XP_002036055.1	XP_017859937.1	XP_001970141.1	XP_002014529.1
Engrailed	NNP_7225059.1	XP_0020911991	XP_002050130.2	XP_002033411.1	x	XP_001976053.1	XP_026841671.1

3. RESULTS AND DISCUSSION

3.1 Phylogeny

The result of phylogenetic analysis of the thirteen proteins in seven different species of the genus *Drosophila* was generated in MEGA XI software. Figs. 1, 2, 3, 4, and 5 depict the evolutionary history inferred using the maximum likelihood (ML) and JTT matrix-based model [29]. The initial trees for the heuristic search were automatically estimated by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances predicted using the JTT model and then picking the topology with the highest log-likelihood value. Figs. 6-10 reveal the evolutionary history using the Maximum parsimony (MP) method. The evolutionary history of the species studied is depicted by a bootstrap consensus tree generated from 1000 repetitions. Next to the branches are the percentage of duplicate trees in which the related taxa are grouped in the bootstrap test (1000 repetitions). The MP tree was created via the Subtree-Pruning-Regrafting (SPR) method at search level 1, in which the starting trees were created by randomly adding sequences (10 replicates). All locations with less than 95 percent site coverage were omitted, meaning that no more than 5% alignment gaps, missing data, or unclear bases were permitted in any position (partial deletion option).

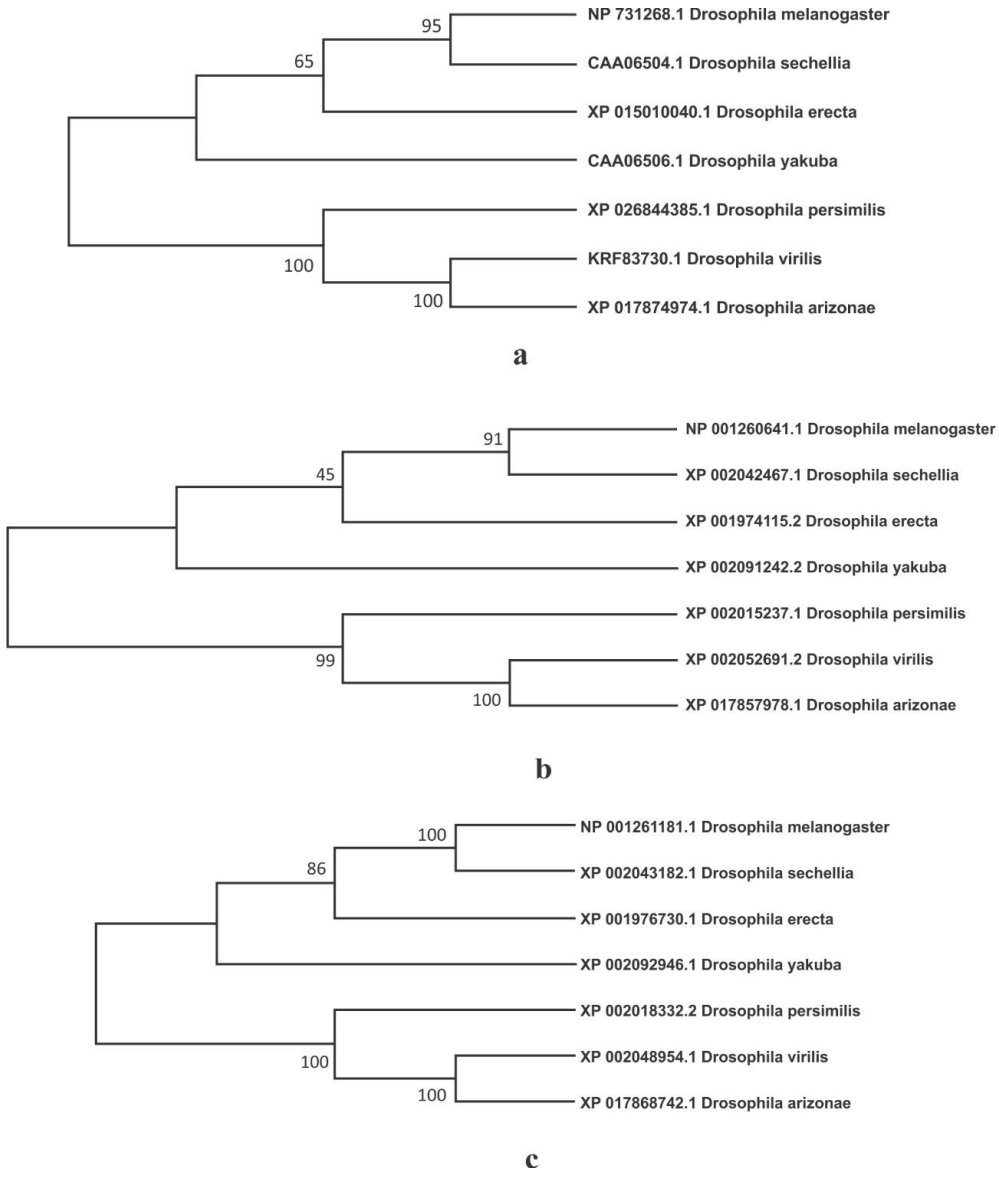

Fig. 1. Showing the phylogenetic relationship of a Hunchback, b Caudal, and c Kruppel among different *Drosophila* species using the Maximum likelihood method

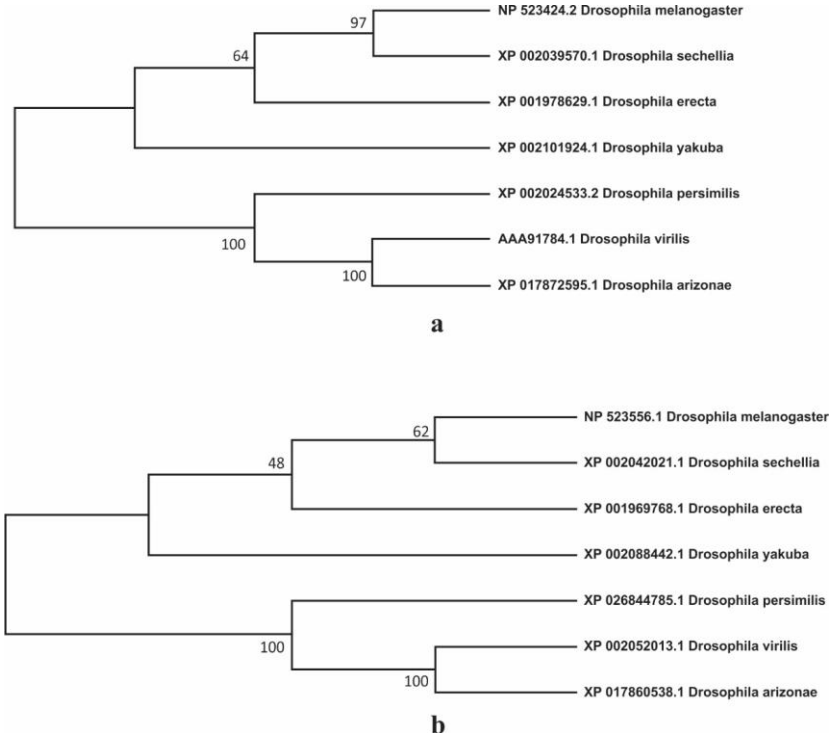

Fig. 2. Showing the phylogenetic relationship of a Runt, and b, Paired among different *Drosophila* species using the Maximum likelihood method

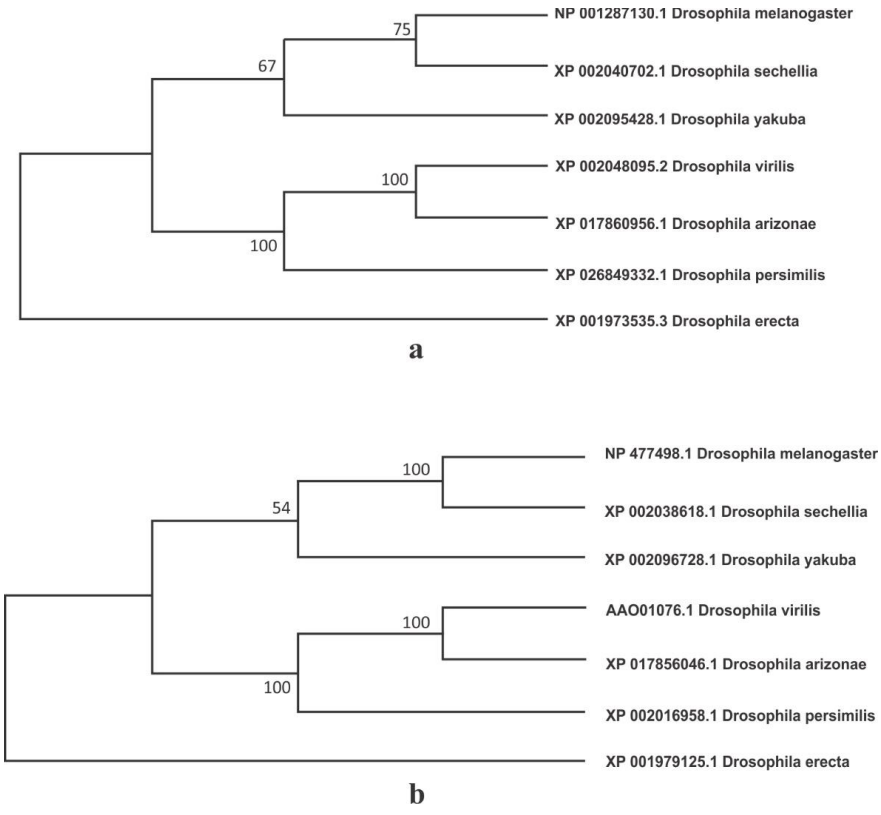

Fig. 3. Showing the phylogenetic relationship of a Knirps, and b Fushi-tarazu among different *Drosophila* species using maximum likelihood method

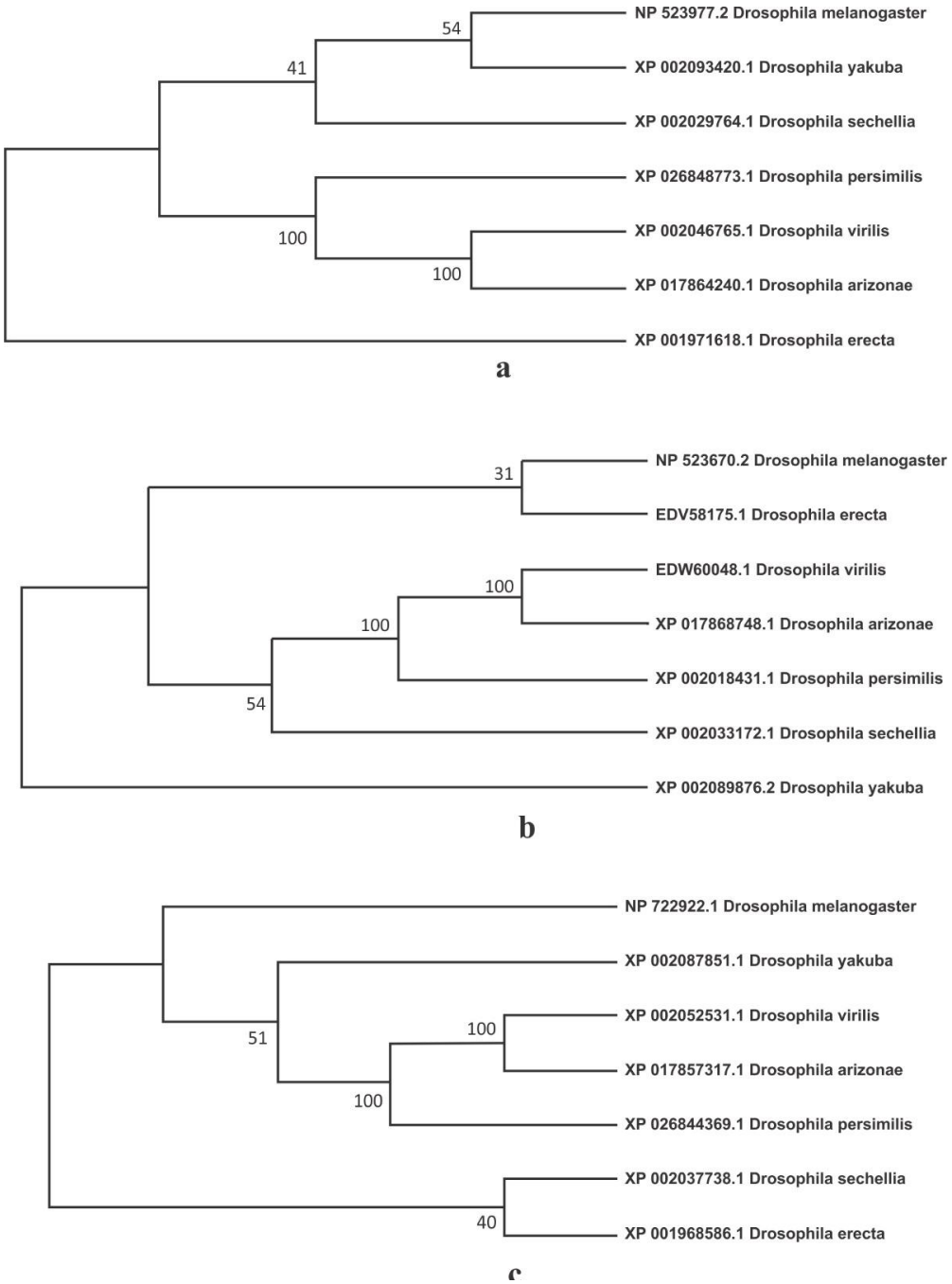

Fig. 4. Showing the phylogenetic relationship of a Hairy, b Even-skipped c Odd skipped, among different *Drosophila* species using maximum likelihood method

For the ML method, the analysis of Phylogenetic trees generated for Hunchback, Kruppel, runt, caudal, and Paired proteins revealed that these proteins diverged earliest in *D. arizonae* and lastly in the *D. melanogaster*. The divergence in the *D. melanogaster* and *D. schellia* occurred simultaneously (Fig. 1 & 2). In contrast, the phylogenetic trees generated for Knirps and Fushi-tarazu proteins showed their late divergence in the *D. melanogaster* and the earliest in the *D. erecta*. The divergence in the *D. virlis* and *D. arizona occur* together and their ancestors have diverged simultaneously with *D. erecta* (Fig. 3). The phylogenetic trees of hairy, even-skipped, odd-skipped, wingless, and engrailed proteins showed different divergence patterns among all *Drosophila* species (Fig. 4 & 5).

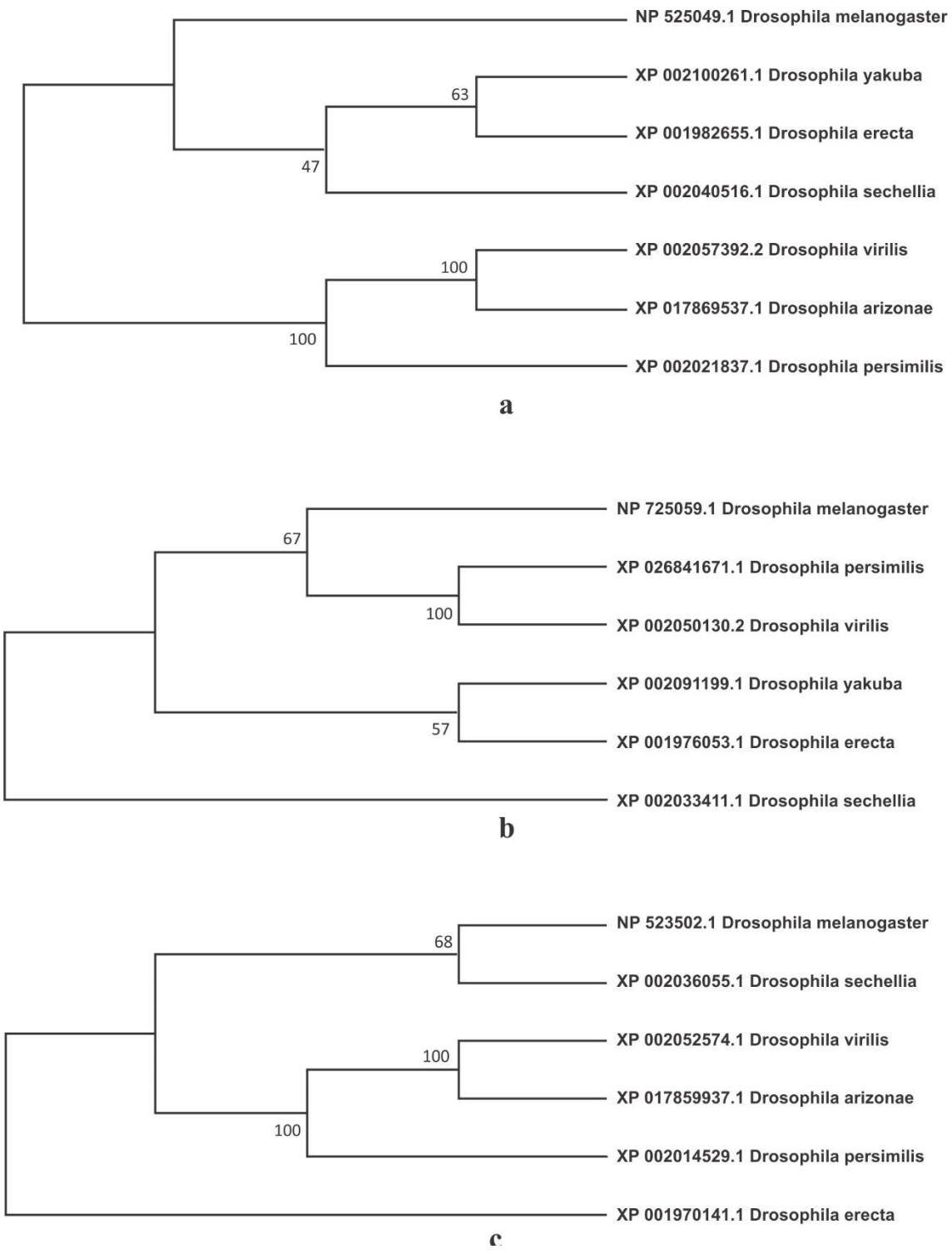

Fig. 5. Showing the phylogenetic relationship of a Giant, b Engrailed, and c Wingless among different *Drosophila* species using the maximum likelihood method

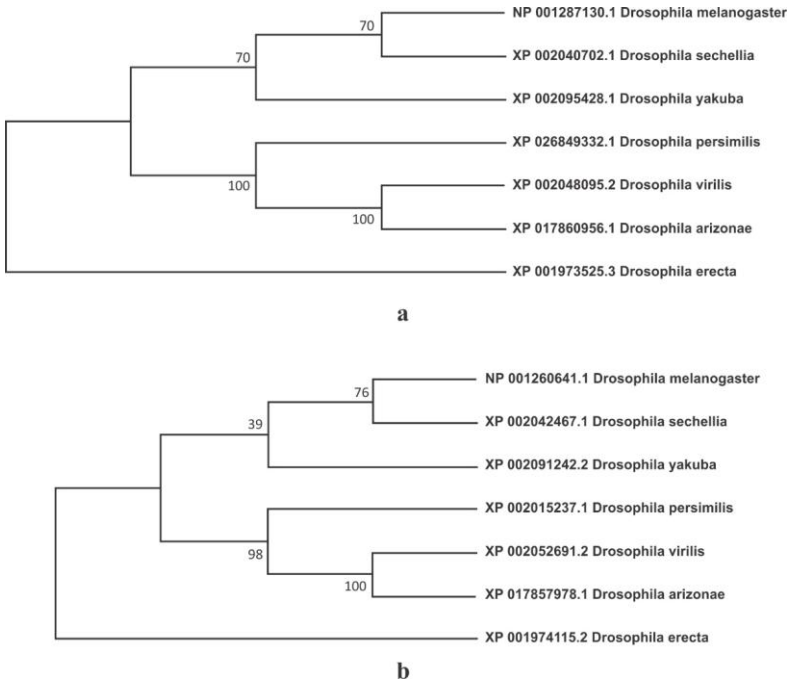

Fig. 6. Showing the phylogenetic relationship of a Knirps and b Caudal proteins in different *Drosophila* species using Maximum parsimony method

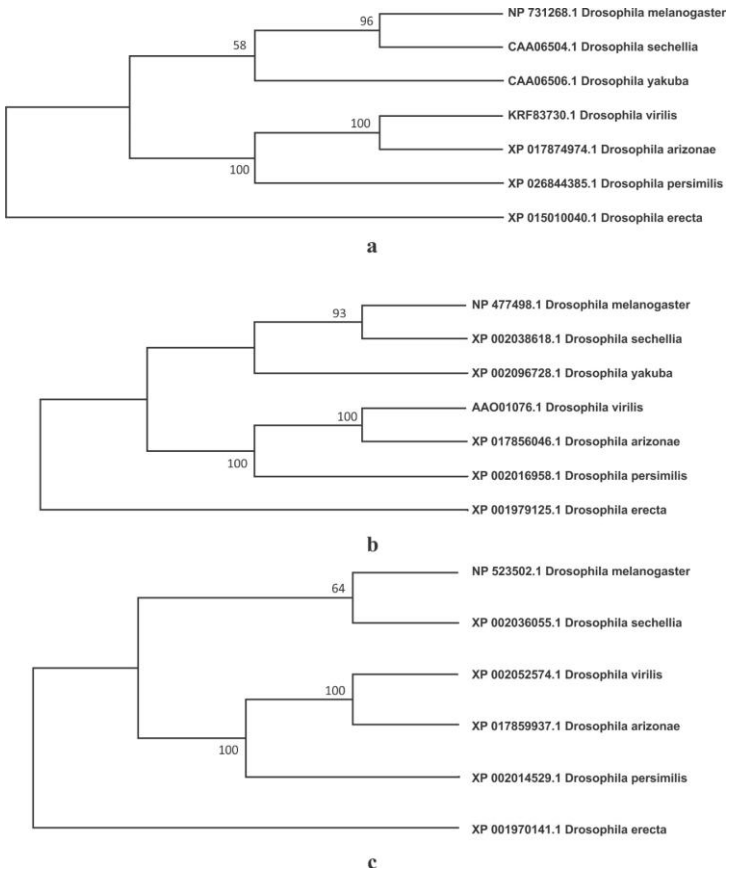

Fig. 7. Showing the phylogenetic relationship of a Hunchback, b Fushi-tarazu and c Wingless proteins in different *Drosophila* species using Maximum parsimony method

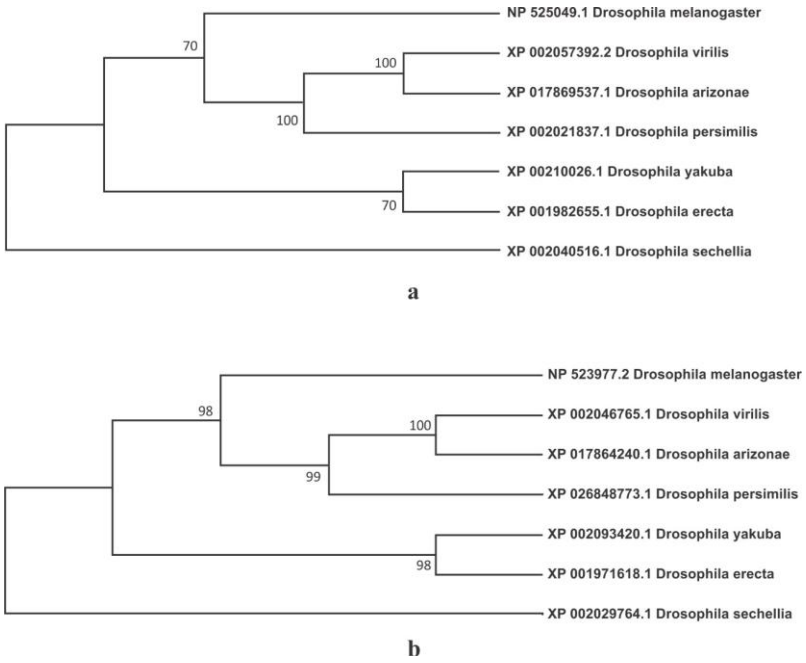

Fig. 8. Showing the phylogenetic relationship of a Giant and b hairy proteins in different *Drosophila* species using the Maximum parsimony method

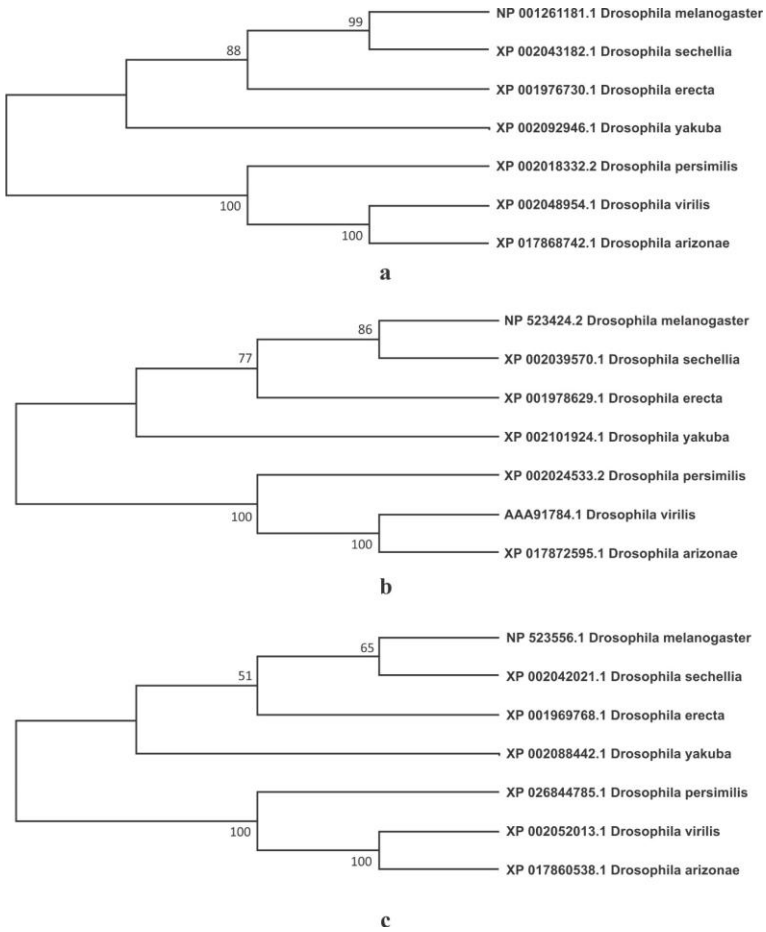

Fig. 9. Showing the phylogenetic relationship of a Kruppel, b Runt, and c Paired proteins in different *Drosophila* species using the Maximum parsimony method

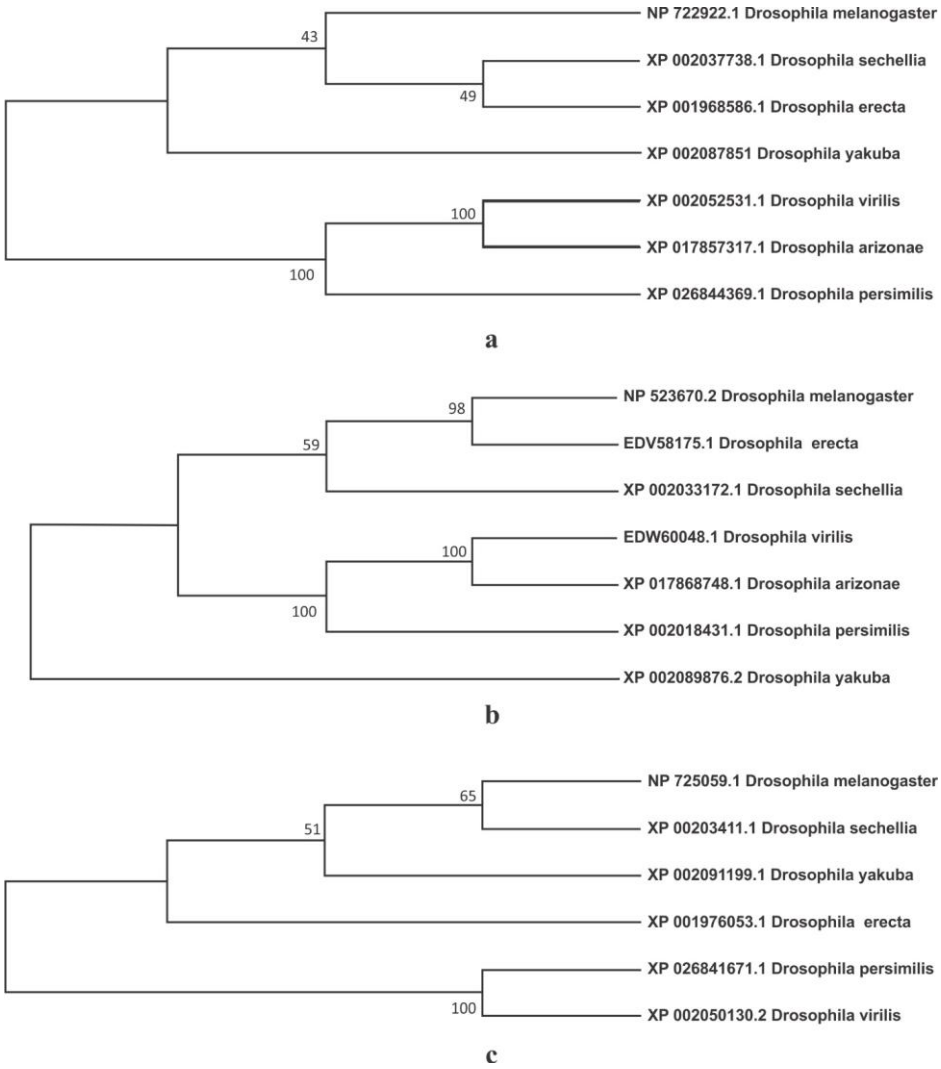

Fig. 10. Showing the phylogenetic relationship of Odd-skipped, b Even-skipped, and c Engrailed proteins in different *Drosophila* species using the Maximum parsimony method

Table 2. Table showing the number of variable sites, parsimony informative sites, and singlet sites detected in MEGA X

Name of gene	Total number of sites	Total Variable sites	Total Parsim informative sites	Total Singlet sites
Hunchback	916	232	127	94
Caudal	466	148	78	87
Kruppel	578	99	64	32
Knirps	609	164	95	56
Giant	546	134	64	63
Hairy	403	82	31	40
Runt	597	169	106	57
Even-Skipped	398	119	51	66
Odd- skipped	453	119	69	49
Paired	694	218	117	94
Fushi-tarazu	528	134	64	63
Wingless	481	70	47	22
Engrailed	599	141	20	109

For the MP method, the phylogenetic tree of Caudal and Knirps revealed that these proteins recently diverged in the *D. melanogaster* and the earliest in the *D. erecta*. The divergence in the *D. virlis* and *D. arizonae* occurred simultaneously (Fig 6) which was similar in Fushi-tarazu, Hunchback, and Wingless, but the divergence of *D. permilis* was different in these tree topologies (Fig 7). The Phylogenetic tree of hairy and Giant showed that these proteins had lastly diverged in the *D. melanogaster* and earliest in the *D. sechellia*. The divergence in the *D. yakuba* and *D. erecta* occurred together and their ancestors had diverged simultaneously with *D. sechellia* (Fig. 8). The analysis of Runt, Paired and Kruppel proteins revealed that these proteins diverged earliest in *D. arizonae* and recently in the *D. melanogaster*. The divergence in the *D. melanogaster* and *D. schellia* occurred simultaneously (Fig. 9). Trees of Odd-skipped, Even-skipped, and Engrailed revealed different divergence patterns among all *Drosophila* species with the exception that among all proteins the *D. melanogaster* is the most recently diverged species. (Fig. 10).

The major reason to study evolutionary genetics is to understand how molecular changes cause morphological changes within and between species. The evolutionary history of *Drosophila* is always of great interest. Till now, many times *Drosophila* phylogeny had been done using molecular data to resolve its evolutionary trends. The evolution of *Drosophila* occurs in the cluster. *Drosophila melanogaster* had a common ancestor with *Drosophila erecta* and *orena* about 12.6 Million Years ago, with *Drosophila yakuba* and *Drosophila teisseri* about 12.8 Million Years ago, with *obscura* group about 54.9 Million Years ago and with *virilis* group 42.9 Million Years ago [30]. To understand the evolutionary trend in *Drosophila,* the foundation was formed by the works of Theockmorton (1975) and Grimaldi (1990). With the help of this, several attempts have been made since then to understand the *Drosophila* tree topology [31]. Morphological and molecular data both had been used to reconstruct the *Drosophila* species tree topology. Remsen and O'Grady (2002) and Schawaroch (2002) reconstructed the species tree topology using morphological data [32] while using molecular data several trees have been constructed using rRNA [32-39] nuclear genes or combined data [40-46].

Seetharam and Stuart (2013), did the species phylogeny of 21 *Drosophila* species [46]. The placement of species in their topology corresponds to the topology predicted in earlier studies [47-48]. Our study revealed that *Drosophila melanogaster* is the most recent diverged species on both ML and MP methods. The phylogenetic tree of Hunchback, Caudal, Kruppel, Runt, and Paired showed a similar tree topology as predicted in earlier studies by, 2007 and Seetharam and Stuart, 2013. These tree topologies are similar to tree topologies of protein Kruppel, Runt, and Paired constructed using the MP method. The divergence of *D. virilis* and *D. arizonae* occur in cluster of all these protein in both ML and MP methods. Surprisingly in the odd skipped tree, constructed using the ML method, the melanogaster group is separated by a wide gap, with *D. sechellia* and *D. erecta* species showing the earliest while *D. melanogaster* and *D. yakuba* have shown recent divergence. In between the *D. persimilis, D. arizonae* and *D. virilis* species can be observed. Similarly, all the other protein tree topologies have conflicted with the previously known trees. The conflicting tree topology may be because comparing orthologous genes for evolutionary analysis can obstruct due to potential horizontal gene transfer, incomplete lineage, and the unrecognized comparison of the paralogous gene [49].

3.2 Sequence Variations

The maximum number of variable sites as shown by the MEGA X software was observed in hunchback, followed by Paired, Runt, and Knirps, and the least number of variations were observed in wingless. The parsimony informative sites were maximum in Hunchback, followed by the Paired, Runt, Knirps, and the least in Engrailed. The details of the total number of variable sites and parsimony sites are given in Table 2. The study of multiple sequence alignment and phylogenetics are useful for the estimation of evolutionary divergence. Multiple sequence alignment analysis leads to an analysis of variable sites and guides phylogenetic analysis. Variable sites are those sites that have at least two different types of amino acids. Within the variable sites, there are parsimony informative (PI) sites. PI sites are those sites that show more than one different amino acid and contain a minimum of two different amino acids that each appears at least in two different sequences [27]. PI sites were important to studying evolutionary history. In the present study, out of total sites, about 14.5% to 31.7% were variable sites. Within these variable sites, except Engrailed, 47.7% to 67.1% were parsimony informative (PI) sites. The maximum variable sites and parsimony sites were found in Hunchback protein while the least was found in the Wingless protein in variable sites while Engrailed protein in the case of parsimony sites. This can be explained by functional constraint which expressed that the more specified functional proteins are subjected to less variation and ultimately to slow evolution [50].

4. CONCLUSION

This study reveals that, among the seven different species of *Drosophila*, *Drosophila melanogaster* was the most recently diverged species in the A-P patterning proteins. Maximum variable sites and parsimony-informative (PI) sites were observed in Hunchback while minimum in Wingless in case of variable sites and Engrailed in parsimony informative (PI) sites.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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