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Rapid Evolution of a Few Members of *Nasuta-Albomicans* Complex of *Drosophila*: Study on Two Candidate Genes, *Sod1* and *Rpd3*

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Abstract *Drosophila nasuta nasuta* ($2n = 8$) and *D. n. albomicans* ($2n = 6$) are morphologically identical, cross fertile and karyotypically dissimilar pair of chromosomal races belonging to *nasuta* subgroup of *immigrans* group of *Drosophila.* Interracial hybridization between these two races yielded karyotypically stabilized newly evolved Cytoraces with new combinations of chromosomes and DNA content, and are called *nasuta-albomicans* complex of *Drosophila*. Along with many other features, striking plasticity in the lifespan has been observed in the karyotypically stabilized members of *nasuta-albomicans* complex of *Drosophila*. These findings provide a strong background to understand any changes at the molecular levels. In view of this, we cloned and characterized *Sod1* and *Rpd3* in the members of *nasuta-albomicans* complex of *Drosophila*. The evolution of *Sod1* and *Rpd3* in *D. n. nasuta* and *D. n. albomicans* is contrasting with the other species of *Drosophila,* at the level of synonymous mutations, intron variation, InDels and secondary structure changes in protein. In the members of NAC of *Drosophila* there were synonymous changes, variations in intron sequences of *Sod1*, whereas, in *Rpd3*, synonymous, nonsynonymous, intron variation, and secondary structure changes in protein were observed. The contrasting differences in the levels of Rpd3 (and Sir2) proteins were also noticed among short-lived and long-lived Cytoraces. The Cytoraces have exhibited not only specific changes in *Sod1* and *Rpd3*, but also show pronounced changes in the levels of synthesis of these proteins, which indicates rapid evolution of these Cytoraces in laboratory. Further these Cytoraces have become a model system to understand the process of anagenesis.

Keywords Evolution · *Nasuta-albomicans* complex · *Drosophila nasuta nasuta* · *Drosophila nasuta albomicans* · Cytoraces · *Sod1* · *Rpd3* · Sir2

Introduction

Drosophila nasuta nasuta ($2n = 8$) consists of a pair of metacentric chromosomes (chromosome 2), two pairs of acrocentric chromosomes (in ♀, chromosome 3 and X, in ♂, chromosome 3 and a submetacentric Y chromosome) with a pair of basic dots (chromosome 4). *D. n. albomicans* ($2n = 6$) has two pairs of metacentric chromosomes (chromosome 2 and X3 in ♀, and Y3 in ♂) with a pair of long dots (chromosome 4). These are a pair of chromosomal races belonging to *nasuta* subgroup of *immigrans* group of *Drosophila* which are morphologically identical, cross fertile and karyotypically dissimilar (Wilson et al. 1969; Kitagawa et al. 1982). Interracial hybridization between these two races yielded karyotypically stabilized four newly evolved: Cytorace 1, (♂, $2n = 7$; ♀, $2n = 6$), Cytorace 2 (in both ♂ and ♀, $2n = 6$), Cytorace 3 (♂, $2n = 7$; ♀, $2n = 8$), and Cytorace 4 (in both ♂ and ♀, $2n = 8$), after F_{20}–F_{50} generations through a phase of karyotypic mosaicism (Ramachandra and Ranganath 1986, 1990). Further, interracial hybridization among the newly evolved four Cytoraces, and the original parental races *D. n. nasuta* and *D. n. albomicans*, resulted in the formation

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of 12 new Cytoraces 5–16. All these karyotypically different recombined genomes were termed as *nasuta-albomicans* complex (NAC) of *Drosophila* (Ramachandra and Ranganath 1996). Based on the karyotypic homology, 16 Cytoraces were grouped under six types (Tanuja et al. 2003).To evolve these Cytoraces in nature it would have taken 1000s of years, whereas, in the laboratory it has taken a decade. During the evolution of these karyotypes some of the parental chromosomes were eliminated and some were retained. The members of NAC of *Drosophila* exhibited noticeable racial divergence in the earlier various studies such as, cytogenetics, morphometric, morphophenotypic, fitness, mating preferences, isozymes, dietary restriction (DR), and life span analysis (Ranganath 2002; Harini and Ramachandra 2003; Ranjini and Ramachandra 2009). Further studies on the members of NAC of *Drosophila* established the evolution of short-lived and long-lived Cytoraces in the laboratory (Ranjini and Ramachandra 2009). The lifespan of *D. n. nasuta*, *D. n. albomicans*, and two short-lived races, Cytorace 3 and Cytorace 15 extended significantly in response to DR when compared to the standard diet. On the other hand, two long-lived Cytoraces (in standard diet), Cytorace 9 and Cytorace 16 did not show any marked difference in their lifespan in response to dietary restriction. We have also reported the differential response to hormesis by laboratory evolved short-lived and long-lived Cytoraces of NAC of *Drosophila* (Ranjini and Ramachandra 2011). These members of NAC of *Drosophila* were not subjected for molecular analysis.

Oxidative stress has been widely implicated as an important factor in the aging process. In *D. melanogaster*, *Sod1* and *Sod2* comprise the total complement of Superoxide dismutase (Sod). Cu, Zn superoxide dismutase, *Sod1* is an eukaryotic cytoplasmic enzyme that performs a protective function in the cell by scavenging superoxide radicals and dismutating them to hydrogen peroxide and molecular oxygen (McCord and Fridovich 1969). Removal of the superoxide radical by Sod and hydrogen peroxide by catalase and glutathione peroxidase prevents formation of the very reactive hydroxyl radical (OH), which is postulated to be responsible for much of the cellular damage (Seto et al. 1987). Mitochondrially localized manganese-binding superoxide dismutase (*Sod2*) is thought to play an important front-line defensive role against aging-related oxidative stress, because mitochondrial respiration is the principal source of reactive oxygen within cells (Kriby et al. 2002). They have reported that ablation of mitochondrial *Sod2* through expression of a GAL4-regulated, inverted-repeat *Sod2* RNA-interference transgene in an otherwise normal animal causes increased endogenous oxidative stress, resulting in the loss of essential enzymatic components of the mitochondrial respiratory chain and the tricarboxylic acid cycle, enhancing sensitivity to applied oxidative stress, causing early-onset mortality in young adults. Extensive genetic analysis of *Sod1* has revealed the critical role of this enzyme in many diverse aspects of the biology of *Drosophila* and is present in all eukaryotes having fairly high rate of evolution and also conserved across a taxa, can be used for phylogenetic analysis (Kwiatowski et al. 1994). Several studies have shown that it is involved in biological process such as determination of adult lifespan and response to oxidative stress (Orr and Sohal 1994; Parkes et al. 1998; Sun and Tower 1999).

Another gene *Rpd3,* (http://flybase.org/) is a histone deacetylase protein coding gene from *D. melanogaster* involved in many unique biological processes such as negative regulation of cellular biosynthetic process, neuron differentiation, embryonic pattern specification, determination of adult lifespan, regulation of RNA metabolic process, coenzyme catabolic process and cellular respiration. Rogina and Helfand (2004) have reported that under DR conditions, activity of the histone deacetylase, *Rpd3* is decreased and the *dSir2*-dependent pathway is activated indicating that *Rpd3* and *Sir2* are the key players in the lifespan extension and DR.

The members of NAC of *Drosophila,* short-lived and long-lived Cytoraces had shown differential responses to DR and oxidative stress. Therefore, we have made an attempt to clone and characterize two candidate genes, *Sod1* and *Rpd3,* to find out any changes at gene and protein levels and to also understand the phylogenetic relationship of the members of NAC of *Drosophila* with other species of *Drosophila.*

Materials and Methods

Experimental Stocks

D. n. nasuta (Coorg, South India, 201.009).

D. n. albomicans (Okinawa, Texas collection, USA 3045.11).

Short-lived Cytoraces: Cytorace 3 (C3), and Cytorace 15 (C15) (Ramachandra and Ranganath 1990, 1996).

Long-lived Cytoraces: Cytorace 2 (C2), Cytorace 9 (C9), Cytorace 11 (C11), and Cytorace 16 (C16) (Ramachandra and Ranganath 1986, 1996).

Isolation and Cloning of *Sod1* and *Rpd3*

Genomic DNA of *D. n. nasuta*, *D. n. albomicans*, Cytoraces 2, 3, 9, 11, 15, and 16 was isolated by using Berkeley *Drosophila* Genome Project (BDGP) protocol with slight modifications. The concentration and purity of genomic DNA was checked using a Biophotometer.

The two sets of primers used for *Sod1* are as follows: Forward primers (1) 5′-ATGGTGGTTAAAGCTGTTTGCG-3′, (2) 5′-GTCGTCTGGTACACCCGTTA-3′, and Reverse

primers (1) 5′-TAACGGGTGTACCAGACGAC-3′, (2) 5′-TTAGATCTTGGCGATGCCG-3′. The primers used for *Rpd3* were: Forward primers (1) 5′-ATGCAGTCCCATAGTAAAAAAC-3′, (2) 5′-ATGCAATCTCATAGCAAAAAGC-3′, and Reverse primers (1) 5′-CTTGATCTTCTCCAGATA-3′, (2) 5′-TCAAATGTTGTTCTCCTTGGCG-3′. PCR amplifications were carried out with the various combinations of primers and except for the annealing temperature, all the other steps remained the same. After amplification, the sizes of PCR products were checked and cloned using InsTAclone™ PCR Cloning Kit from Fermentas (Catalogue No. K1214).

Analysis of Cloned Genes

Sequence analyses of cloned genes, *Sod1* and *Rpd3* were done by using CLUSTAL W, NCBI BLAST, ACCELRYS GENE, MEGA version 4 (Tamura et al. 2007), and DnaSP V5 (Librado and Rozas 2009). The Tajima's test (Tajima 1989) was performed to distinguish whether DNA sequences are evolving randomly (neutrally) or evolving under a non-random process, including directional/balancing selection, demographic expansion or contraction, genetic hitchhiking or introgression by utilizing transition and transversion data and the ratio at first, second and third position of codon. In the Tajima's test, p value less than 0.05 was used to reject the null hypothesis of equal rates between lineages.

Codon usage bias was analyzed in both the genes by using DnaSP v5. The analysis included Effective Number of Codons-ENC (Wright 1990) and Codon Bias Index (CBI) which is a measure of the deviation from the equal use of synonymous codons. CBI values range from 0 (uniform use of synonymous codons) to 1 (maximum codon bias), Scaled Chi Square ($SChi^2$) (Shields et al. 1988) is a measure based on the Chi square statistics; i.e., based on the difference between the observed number of codons and those expected from equal usage of codons.

Assay on Rpd3 and Sir2 Proteins

Quantification of Rpd3 and Sir2 was done through ELISA by using primary antibodies—Rpd3 (dN-12): sc-30557 and—Sir2 (dF-16): sc-27476 (Santa Cruz Biotechnology, INC.). Sample preparation of both male and female flies (each 20) of Cytoraces 3, 15, 9, and 16 were fed with the standard diet ($\sim$15 mg of yeast) and restricted diet ($\sim$2 mg of yeast) was made by using the modified protocol of Yu et al. (1999). ELISA was performed by slight modification of protocol from http://www.abcam.com/. For ELISA experiment three replicate assessments were carried out with 40 flies (20 flies each for both male and female) of Cytoraces, 3, 15, 9 and 16. Standard curve was drawn from the data obtained from the serial dilutions with concentration on the x-axis versus absorbance on the y-axis. The concentration of test samples was obtained by plotting the optical density value perpendicular to the standard curve. Mean concentration ± Standard error was calculated using SPSS software and the graph was generated accordingly. One-Way ANOVA was carried out using SPSS software version 10.0 to understand the significance. A value of less than 0.05 was set as the minimum level of significance.

Results

Isolation and Cloning of *Sod1* and *Rpd3*

In the present study, degenerate primers specific for *Sod1* and *Rpd3* were created by using the conserved sequences of 12 *Drosophila* species genomes and were PCR amplified. The amplified clones of *Sod1* and *Rpd3* were cloned and sequenced in all the members selected for the present study. The two exons of *Sod1* were found interspersed with an intron and a number of deletions and insertions were found in the other members of NAC of *Drosophila* (Fig. 1a). Schematic representation of the organization of *Rpd3* and the primers designed for amplifications are presented in Fig. 1b. The accession number of cloned sequences of *Sod1* and *Rpd3* of the above members under study and the 12 *Drosophila* species genome sequenced is presented in Table 1.

Analysis of *Sod1*

The synonymous substitutions in entire transcript of *Sod1* among *Drosophila* stocks under study were analyzed (Supplementary Table 1). *D. mojavensis* showed the highest number of substitutions with all the *Drosophila* species. *D. n. nasuta*, *D. n. albomicans* and the Cytoraces showed the highest number of synonymous substitutions with *D. obscura* group, and the lowest with *D. sechellia* and *D. yakuba* of *D. melanogaster* subgroup. The maximum number of synonymous substitutions was seen in *D. n. nasuta* and *D. n. albomicans* as compared to C2, C9, C16. When C2, C9, and C16 were compared with C3, C11, and C15, only one synonymous substitution was observed. The nonsynonymous substitutions in entire transcript of *Sod1* between *Drosophila* stocks under study was analysed (Supplementary Table 2). There were no nonsynonymous substitutions among *D. n. nasuta*, *D. n. albomicans* and Cytoraces under study; however, these races showed the highest number of nonsynonymous substitutions with *D. ananassae* and the lowest with *D. grimshawi*.

Figure 2a depicts the codon usage bias in *Sod1* of all the *Drosophila* stocks under study. The highest Effective number of codons (ENC) and Scaled Chi square ($Schi^2$)

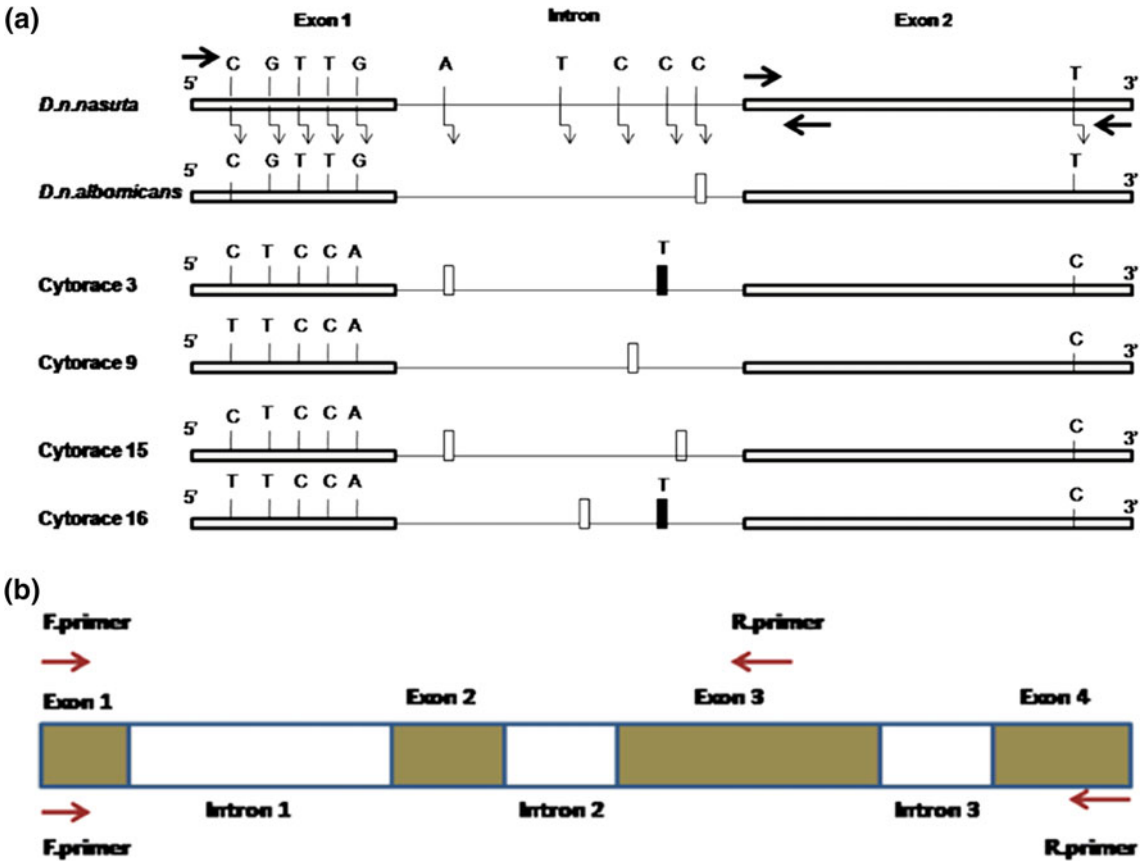

Fig. 1 **a** Schematic representation of genomic organization of *Sod1* in *D. n. nasuta* and other members of NAC of *Drosophila*. *Filled blocks* indicate insertion and empty blocks indicates deletion. The *bold arrows* indicate primer regions. The *highlighted nucleotide* in each member represents the substitution while treating *D. n. nasuta* as standard. **b** Schematic representation of organization of *Rpd3*. *Arrows* indicate the direction of primers used to clone Rpd3 in the members of NAC of *Drosophila* under study

were observed in *D. mojavensis* and *D. pseudoobscura* respectively, while the highest Codon bias index (CBI) and G+C content at third codon position (G+C3s) were seen in *D. yakuba.* Among the members of NAC of *Drosophila*, ENC was the highest, while CBI, Schi^2 and G+C3s were the lowest in C2, C9, and C16. Table 2 presents the Tajima's relative rate test based on transitions and transversions change in *Sod1* by using *D. melanogaster* as an outgroup. Transition and transversion changes were not observed between *D. n. nasuta* and *D. n. albomicans*. The highest numbers of bases in intron of *Sod1* was observed in *D. melanogaster* species group ranging from 708 to 731 bp, and the lowest number of bases in intron were observed in *D. obscura* group (Table 3). Compared to *D. n. nasuta,* all these Cytoraces have shown insertions and deletions in intronic region as depicted in Fig. 1a.

The multiple alignment of Sod1 protein for all the *Drosophila* species under study was obtained by Accelrys gene software (Fig. 3). There were no aminoacid changes between *D. n. nasuta* and *D. n. albomicans*. However, both of them showed maximum amino acid changes (22) with *D. ananassae*. Similar to *D. n. nasuta* and *D. n. albomicans*, *D. pseudoobscura* and *D. persimilis* being in the same group had not shown any aminoacid change. These aminoacids changes showed change in helix, sheet and band in *D. n. nasuta* when compared to other *Drosophila* species. The chemical and functional changes in the aminoacids display the secondary structure changes (data not shown).

Analysis of *Rpd3*

The schematic representation of *Rpd3* having four exons and three introns is presented in Fig. 1b. The synonymous substitutions in entire transcript of *Rpd3* among *Drosophila* species under study is shown in Supplementary Table 3. *D. willistoni* showed the highest substitutions rate with all the *Drosophila* species. The total length of the *Rpd3* in *D. n. nasuta* was 2138 bp and four insertions were seen in the intron of C9, while all other races showed similar intron length when compared to *D. n. nasuta*. *D. n. nasuta*, *D. n. albomicans* and Cytoraces under study showed the highest synonymous substitutions with *D. wllistoni*, and the lowest with *D. grimshawi*. When *D. n. nasuta* and *D. n.*

Table 1 List of *Drosophila* species and members of *nasuta-albomicans* complex (NAC) of *Drosophila* used for the analysis of *Sod1* and *Rpd3* with their respective NCBI GenBank accession numbers

Species and races	*Sod1*	*Rpd3*
D. melanogaster	NM_057387.4	NM_139661.3
D. simulans	XM_002084421.1	XM_002083624.1
D. sechellia	XM_002030033.1	XM_002035338.1
D. yakuba	XM_002094339.1	XM_002093695.1
D. erecta	XM_001972313.1	XM_001971889.1
D. ananassae	XM_001956458.1	XM_001956151.1
D. pseudoobscura	XM_001353908.2	XM_001352613
D. persimilis	XM_002024032.1	XM_002028680.1
D. willistoni	XM_002068559.1	XM_002062520.1
D. mojavensis	XM_002008745.1	XM_002007045.1
D. virilis	XM_002048496.1	XM_002060001.1
D. grimshawi	XM_001985159.1	XM_001983217.1
D. n. nasuta	FJ554532.2	HQ323752.1
D .n. albomicans	GU300109.2	HQ326584.1
Cytorace 2	HQ326578.1	HQ379189.1
Cytorace 3	HQ326579.1	HQ379190.1
Cytorace 9	HQ326580.1	HQ379191.1
Cytorace 11	HQ326581.1	HQ379192.1
Cytorace 15	HQ326582.1	HQ379193.1
Cytorace 16	HQ326583.1	HQ379194.1

albomicans were compared with the other members of NAC of *Drosophila,* C9, C15, and C16 showed 22, 1 and 2 bp synonymous substitutions respectively. The nonsynonymous substitutions in the transcript of *Rpd3* between *Drosophila* species under study is presented in Supplementary Table 4. Among the members of NAC of *Drosophila*, C3, C15, and C16 showed 1, 3 and 2 bp substitutions respectively. The members of NAC of *Drosophila* showed the highest number of substitutions with *D. mojavensis* and the lowest with *D. virilis*.

Figure 2b depicts the codon usage bias in *Rpd3* of all the members under study. The highest ENC was observed in *D. willistoni* and the lowest in *D. ananassae*, *D. virilis*, and *D. ananassae* showed the highest $Schi^2$ and G+C3s. *D. willistoni* showed the lowest CBI and $Schi^2$ than other *Drosophila* species. Among the members of NAC of *Drosophila,* all have shown almost similar codon usage values than other *Drosophila* species under study. Table 4 represents the data obtained by Tajima's relative rate test based on transitions and transversions change in *Rpd3* by using *D. melanogaster* as an outgroup. However, significant rejection of null hypothesis was seen between *D. n. nasuta* and other *Drosophila* species. Transition and transversion changes were not observed between *D. n. nasuta* versus *D. n. albomicans*, C2 and C11. The highest and the lowest number of bases in intron of *Rpd3* were observed in *D. virilis* and *D. grimshawi* respectively than other *Drosophila* species. The intron length of *D. n. nasuta* was longer than *D. obscura and D. grimshawi*, but shorter than other *Drosophila* species under study (Table 3). Among the members of NAC of *Drosophila*, the intron length of C9 was four bases more (585 bp) than all other members of NAC of *Drosophila* (581 bp).

As indicated in Table 5, the members of NAC of *Drosophila* under study had a shorter length of protein with 518 aminoacids compared to all other *Drosophila* species. *D. n. nasuta* and *D. n. albomicans* did not show any aminoacid changes between them. However, both of them showed more amino acid changes with *D. mojavensis* and less with *D. virilis*. Due to this, changes in helix, sheet and band were observed in *D. n. nasuta* when compared to other *Drosophila* species. The chemical and functional changes in the aminoacids display the secondary structure changes (data not shown). Three amino acid changes, A472 V, N485D and S492I were seen in C15. The split band was seen in turn region of secondary structure of Rpd3 in C15. In C16, two aminoacid changes, Q109P and R429G were noticed, due to which, in helix region, there was one bigger band and loss of one band, while, in turn region, one extra band was noticed (data not shown). There was only one aminoacid change, G506S in C3 which did not cause any secondary structure variation.

Figure 4 represents evolutionary relationships of aminoacid sequence in Sod1 of all the members under study with *Aedes aegypti* as an outgroup. The members of *D. melanogaster* subgroup formed a separate lineage, where *D. melanogaster*, *D. simulans* and *D. sechellia* formed a single clade giving rise to sister clades comprising of *D. erecta* and *D. yakuba*. *D. ananassae* formed a sister clade with two members of *D. obscura.* This clade is then branched with *D. willistoni*. All the eight members of NAC of *Drosophila* formed separate clade branched with *D. grimshawi*, and they showed 72 % homology with *A. aegypti*. Figure 5 represents evolutionary relationships of aminoacid sequence in Rpd3 of all the members under study with *A. aegypti* as an outgroup. A big clade was formed comprising of *D. melanogaster* group, *D. ananassae* and *D. obscura* group. The Nei-Gojobori tree in track with *D. willistoni* formed two clades comprising of *D. grimshawi* and *D. virilis,* and another clade comprised of eight members of NAC of *Drosophila*, where *D. n. nasuta, D. n. albomicans*, C2, C9, C11and C16 shared single clade, and C3, and C15 formed separate clades. The members of NAC were closer to *D. grimshawi*, and they showed 89 % homology with *A. aegypti*. *D. mojavensis* with more number of aminoacid changes formed a distinct clade connected to both subclades.

Estimation of Rpd3 and Sir2 proteins

Mean concentration ± Standard error of Rpd3 and Sir2 protein in C3, C9, C15, and C16 from the three replicates

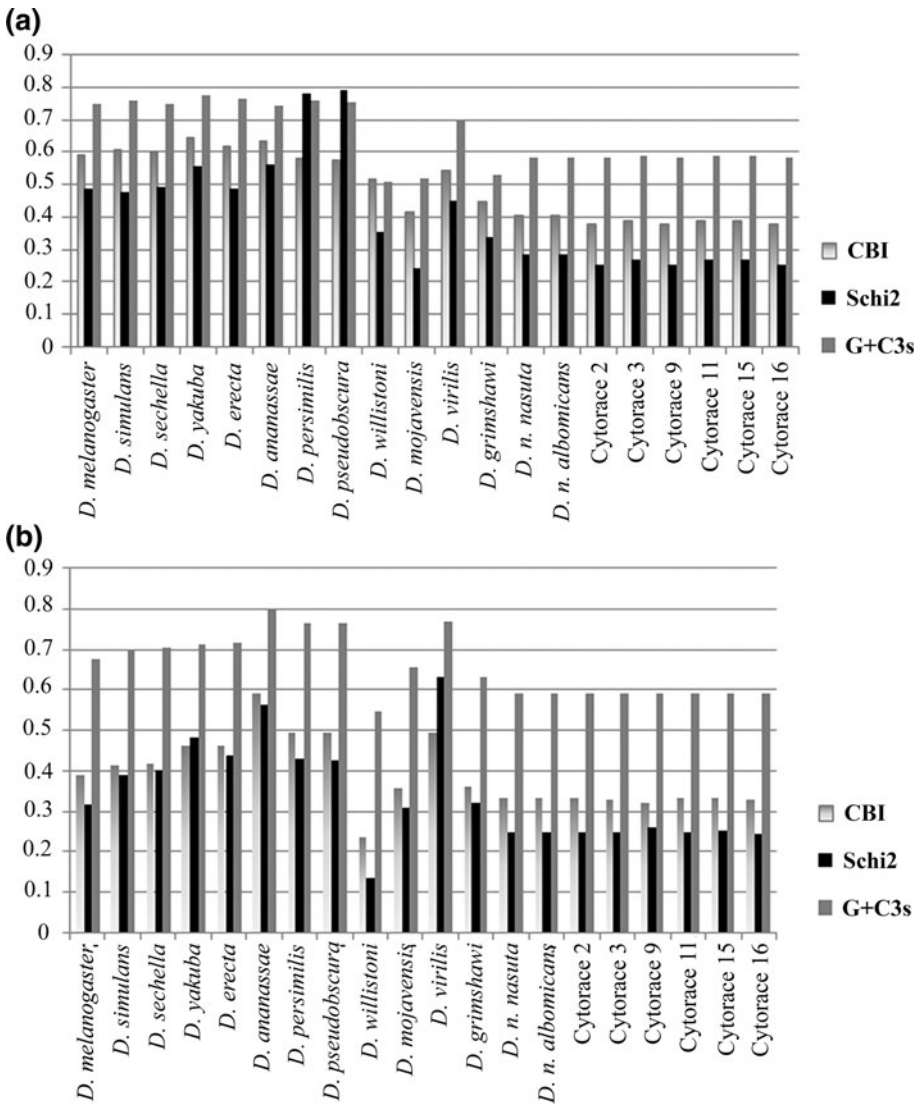

Fig. 2 Codon Usage bias in *Sod1* (**a**) and *Rpd3* (**b**) of all the 12 *Drosophila* species and 8 members of NAC of *Drosophila*. *CBI* Codon bias index, $SChi^2$ Scaled Chi square, G+C3s = G+C content at third codon position

was presented in Table 6. The mean concentration of Rpd3 protein in short-lived Cytoraces, C3 and C15 were significantly higher in control than DR ($p < 0.005$), whereas, there was no remarkable difference between control and DR in long-lived Cytoraces, C9 and C16 (Fig. 6a). The mean concentration of Sir2 protein in short-lived Cytoraces, C3 and C15 was higher in DR control ($p < 0.005$). However, there was no remarkable difference between control and DR dietary restriction in long-lived Cytoraces, C9 and C16 (Fig. 6b). In both Rpd3 and Sir2, there were no significant differences between the short-lived Cytoraces, C3 and C15 and long-lived Cytoraces, C9 and C16.

Discussion

The role of hybridization in evolution has been debated for over a century. Hybridization and introgression have been neglected in evolutionary biology since the 1940s. The natural hybridization can affect the evolutionary history of the groups in which it occurs (Arnold 1996). The recombinant hybrids are less fit on average; some gene combinations may be fitter than the parents, even in the parental environment (Barton 2001). Recent molecular genetic studies indicate that hybridization is surprisingly frequent in natural populations and it may allow populations to regain traits that have been lost and possibly to replace damaged alleles with functional copies from related species (Rieseberg 2009). Hybrid or "recombinational" speciation is one of the suggested pathways by which new species might arise rapidly via hybridization between chromosomally or genetically divergent parental species (Ungerer et al. 1998). In the present study, the hybrids, karyotypically stabilized 700 generations old, cytoraces evolved through "recombinational" speciation (Harini and Ramachandra 2003) and their parents were analysed to check the molecular divergence if any in two genes *Sod1* and *Rpd3*.

Table 2 The equality of evolutionary rate between *D. n. nasuta*[a] and *Drosophila* species/races tested using *D. melanogaster*[c] as an outgroup in Tajima's relative rate test in MEGA4 for *Sod1*

Species/races	Identical sites	Divergent sites	Unique differences in			χ^2	*p* value
			Sequence[a]	In sequence[b]	Sequence[c]	DF = 2	
D. n. nasuta[a] versus *D. simulans*[b]	354	5	94	2	4	88.17	0.0001*
D. n. nasuta[a] versus *D. sechellia*[b]	354	5	91	2	7	85.17	0.0001*
D. n. nasuta[a] versus *D. yakuba*[b]	350	6	87	6	10	70.55	0.0001*
D. n. nasuta[a] versus *D. erecta*[b]	350	7	88	6	8	71.53	0.0001*
D. n. nasuta[a] versus *D. ananassae*[b]	330	14	66	26	23	17.39	0.0001*
D. n. nasuta[a] versus *D. pseudoobscura*[b]	322	20	53	31	30	5.76	0.016*
D. n. nasuta[a] versus *D. persimilis*[b]	322	20	53	31	30	5.76	0.016*
D. n. nasuta[a] versus *D. willistoni*[b]	323	20	43	33	40	1.32	0.251
D. n. nasuta[a] versus *D. mojavensis*[b]	318	18	38	38	47	0.00	1.000
D. n. nasuta[a] versus *D. virilis*[b]	328	23	47	28	33	4.81	0.028*
D. nasuta[a] versus *D. grimshawi*[b]	314	21	30	42	52	2.00	0.157
D. n. nasuta[a] versus *Cytorace 2*[b]	356	1	5	0	97	5.00	0.025*
D. n. nasuta[a] versus Cytorace 3[b]	356	1	4	0	98	4	0.045*
D. n. nasuta[a] versus Cytorace 9[b]	356	1	5	0	97	5.00	0.025*
D. n. nasuta[a] versus Cytorace 11[b]	356	1	4	0	98	4	0.045*
D. n. nasuta[a] versus Cytorace 15[b]	356	1	4	0	98	4	0.045*
D. n. nasuta[a] versus Cytorace 16[b]	356	1	5	0	97	5.00	0.025*

* Significant at 5 % and above

[a] *D. n. nasuta*

[b] Other *Drosophila* species

[c] *D. melanogaster*

Table 3 Total number of nucleotide bases lost and gained in the intron of *Sod1* and *Rpd3* in *Drosophila* species when compared with *D. n. nasuta*

Species	*Sod1*			*Rpd3*		
	Total intron length (in bp)	Intronic bases lesser than *D. n. nasuta*	Intronic bases more than *D. n. nasuta*	Total intron length (in bp)	Intronic bases lesser than *D. n. nasuta*	Intronic bases more than *D. n. nasuta*
D. n. nasuta	408			581		
D. persimilis	330	78		552	29	
D. pseudoobscura	330	78		551	30	
D. melanogaster	725	–	317	610		29
D. simulans	730	–	322	616		35
D. sechellia	731	–	323	595		14
D. yakuba	708	–	300	615		34
D. erecta	709	–	301	621		40
D. ananassae	624	–	216	701		120
D. grimshawi	462	–	54	537	44	
D. mojavensis	614	–	206	703		122
D. virilis	553	–	145	1497		916
D. willistoni	417	–	9	921		340

Sod1 and *Rpd3* in *Drosophila* Species

Despite the large number of genetic and genomic resources, little is known concerning the phylogenetic relationships, ecology, and evolutionary history of all but a few species of *nasuta* subgroup of *immigrans* group of *Drosophila* (Katoh et al. 2007). In the present study, the evolution of *Sod1* and *Rpd3* in *Drosophila* lineage is very

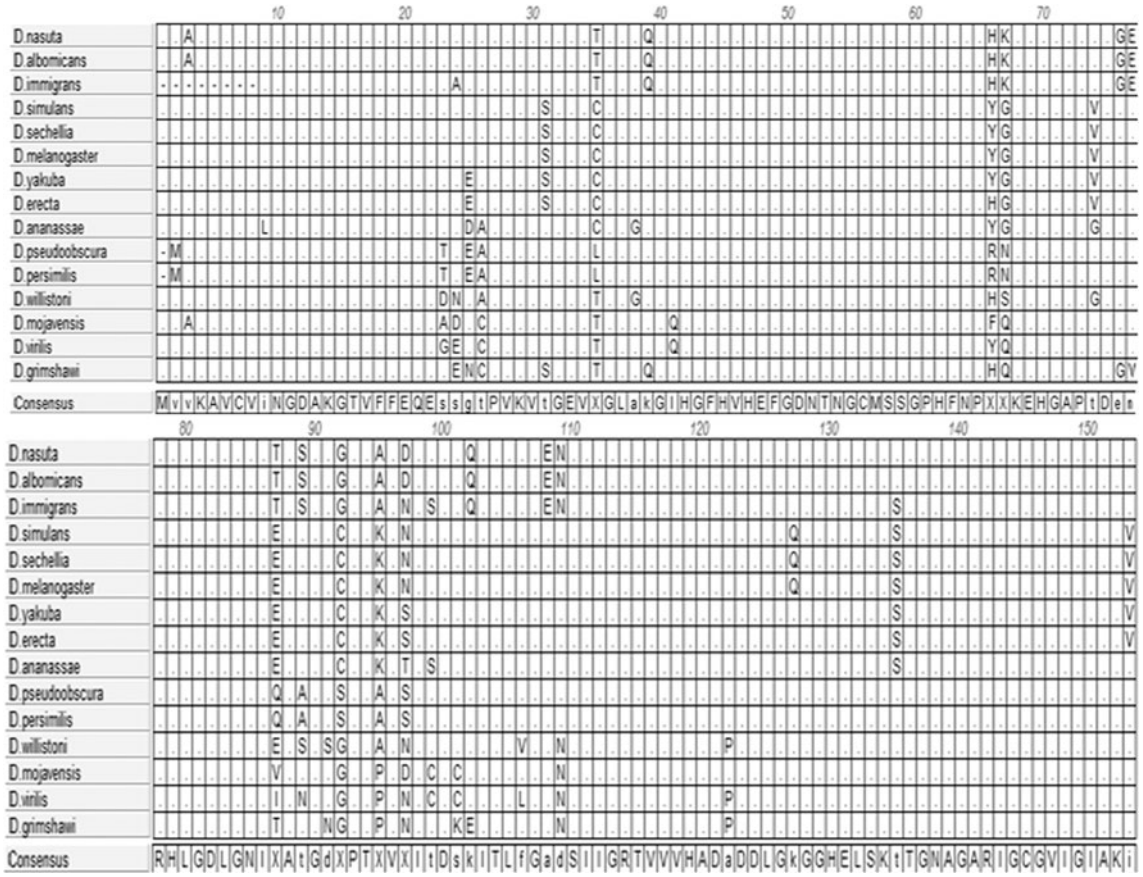

Fig. 3 Multiple alignment of Sod1 protein in 14 *Drosophila* species using Accelrys gene software. *Dots* in the alignment denote identical aminoacids in different species

interesting. With respect to synonymous substitutions in *Sod1, D. n. nasuta* has shown closeness with *D. melanogaster* group, whereas *Rpd3* showed consistent positioning of *D. n. nasuta* with *virilis-repleta* group as of nonsynonymous substitutions. Both *Sod1* and *Rpd3* data revealed the clustering of *D. n. nasuta* with v*irilis-repleta* group with respect to transition and transversion, synonymous versus nonsynonymous substitutions, codon bias, and GC content at third position of codon suggesting the weak purifying selection and a large fraction of synonymous changes behaving as neutral. These findings indicate that selection is relaxed in the *Drosophila* lineage and strongly support the nearly neutral character of nonsynonymous mutations.

Coding Sequence of Sod1 and Rpd3

Throughout the *Drosophila* lineage, only one aminoacid deletion is observed in *Sod1* of *D. obscura* group, whereas, in *Rpd3* more InDel diversity have been observed across the species. *D. n. nasuta* has the less aminoacids than other species. Even though more number of InDels are observed in *Rpd3*, the substitution of aminoacid has not caused much change in the secondary structure and physiochemical features as compared to *Sod1*. This could be due to the substitutions of amino acid in the nonconserved regions of proteins that are nearly neutral or slightly positively selected (Freese and Yoshida 1965; Margoliashand Smith 1965; Zuckerkandl 1965).

Introns in Sod1 and Rpd3

Hawkins (1988) stated that in contrast to the vertebrates, which typically have large genomes and genes punctuated by large introns, dipterans have compact genomes with few and small introns. Kwiatowski et al. (1994) reported evolutionary favoring deletion of the second intron of *Sod1* in the *Drosophila* lineage, and suggested that there is a general trend in the evolution of the *Diptera* favoring intron size reduction and even elimination. Similarly, Roy and Penny (2007) noted that certain ancient lineages underwent big bursts of intron gain while subsequent lineages experienced a precipitous decline in intron gain rates. The intron DR of *Cu*, *Zn Sod1* in the two very closely related species, *D. melanogaster* and *D. simulans* found that nucleotide deletion/insertion is much more prevalent than

Table 4 The equality of evolutionary rate between *D. n. nasuta* and *Drosophila* species/Races tested using *D. melanogaster*[c] as an outgroup in Tajima' relative rate test in MEGA4 for *Rpd3*

Species/Races	Identical sites	Divergent sites	Unique differences in sequence[a]	Unique differences in sequence[b]	Unique differences in sequence[c]	χ^2 (DF = 2)	*p* value
D. n. nasuta[a] versus *D. simulans*[b]	1250	12	246	14	14	207.02	0.0001*
D. n. nasuta[a] versus *D. sechellia*[b]	1247	11	245	17	16	198.41	0.0001*
D. n. nasuta[a] versus *D. yakuba*[b]	1236	12	225	28	35	153.40	0.0001*
D. n. nasuta[a] versus *D. erecta*[b]	1239	17	232	25	23	166.73	0.0001*
D. n. nasuta[a] versus *D. ananassae*[b]	1179	49	152	85	71	18.94	0.0001*
D. n. nasuta[a] versus *D. pseudoobscura*[b]	1170	51	136	94	85	7.67	0.005*
D. n. nasuta[a] versus *D. persimilis*[b]	1171	50	136	93	86	8.07	0.004*
D. n. nasuta[a] versus *D. willistoni*[b]	1124	62	102	140	108	5.97	0.014*
D. n. nasuta[a] versus *D. mojavensis*[b]	1132	60	101	131	109	3.88	0.048*
D. n. nasuta[a] versus *D. virilis*[b]	1180	41	103	84	128	1.93	0.164
D. nasuta[a] versus *D. grimshawi*[b]	1167	50	77	97	145	2.30	0.129
D. n. nasuta[a] versus Cytorace 3[b]	1263	0	0	1	272	1.00	0.317
D. n. nasuta[a] versus Cytorace 9[b]	1258	5	11	6	256	1.47	0.225
D. n. nasuta[a] versus Cytorace 15[b]	1261	1	0	3	271	3	0.083
D. n. nasuta[a] versus Cytorace 16[b]	1262	1	1	2	270	0.33	0.563

* Denotes significant at 5 % and above

[a] Denotes *D. n. nasuta*

[b] Other *Drosophila* species

[c] *D. melanogaster*

substitution. In congruence with this, in the present study, reduction in intron length has been noticed in *Drosophila* lineage. The intron diversity places subgenus *Sophophora* before subgenus *Drosophila*. Surprisingly, *D. pseudoobscura*, *D. persimilis* and *D. willistoni* which forms closer clade with *D. melanogaster* with nucleotide substitutions at coding region have half the length of intron which is the smallest intron size as compared to all other *Drosophila* species under study indicating closeness of *D. n. nasuta* to these species. However, *D. n. nasuta* with the intron length of 408 bp falls after *virilis-repleta* group in the *Drosophila* lineage which is consistent with nucleotide substitution rate at coding region. The evolution of *Rpd3* intron is very fascinating with respect to variations in length in different *Drosophila* species under study. The maximum length of intron was seen in *D. virilis* and all species under study except *D. persimilis, D. pseudoobscura*, and *D. grimshawi* showed an increased intronic length.

Evolutionary Dynamics of *Sod1* and *Rpd3* in Few Members of NAC of *Drosophila*

D. n. albomicans and other members of NAC of *Drosophila* have not shown any nonsynonymous substitutions in *Sod1*. Though synonymous substitutions are not seen between *D. n. nasuta* and *D. n. albomicans,* on comparison to Cytoraces, C2, C9, and C16 formed separate branching and C3, C11, and C15 formed another branch with specific synonymous substitutions. The C2, C9, and C16 with low CBI than any other *Drosophila* under study, have shown minimum codon bias. The data by Tajima's relative test based on transition and transversion supported the neutral selection between *D. n. nasuta* and the members of NAC of *Drosophila.* With respect to *Rpd3*, C9 has shown the highest synonymous substitutions with other members, and, C3, C15, and C16 have shown prominent nonsynonymous substitutions with aminoacid changes which affected the secondary structure of Rpd3 protein. The *Rpd3* of C3 and C9 showed more codon bias than any other *Drosophila* species under study. The *Rpd3* of C9 is the one among NAC of *Drosophila* which is undergoing purifying selection.

Based on *Sod1* phylogeny reported by Kwiatowski et al.(1994), within ~2.3 MY, number of synonymous substitutions in *Sod1* between *D. melanogaster* and *D. simulans* are 11 and the difference in ENC, CBI and G+C3s are 3, 2.2 and 2 % respectively. There are no nonsynonymous and aminoacid substitutions between them. Interestingly, among the members of NAC of *Drosophila* C2, C9, and C16 as well as C3, C11, and C15 have

Table 5 Total number of amino acids in *Rpd3* of *D. n. nasuta,* when compared to different *Drosophila* species

Species	Total length of aminoacids	Less number of aminoacids in *D. n. nasuta*
D. n. nasuta	518	
D. persimilis	530	12
D. p. pseudoobscura	530	12
D. grimshawi	559	41
D. melanogaster	521	3
D. simulans	521	3
D. sechellia	521	3
D. yakuba	521	3
D. erecta	521	3
D. ananassae	525	7
D. mojavensis	525	7
D. virilis	527	9
D. willistoni	531	13

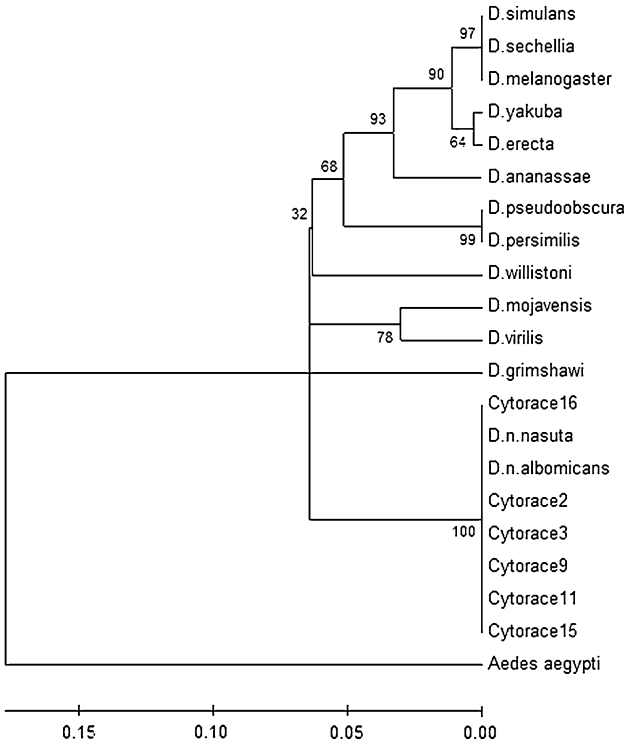

Fig. 4 The evolutionary distances were computed using the Nei-Gojobori method and are in the units of the number of aminoacid substitutions per site in Sod1. The evolutionary history was inferred using the Neighbor–Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA4

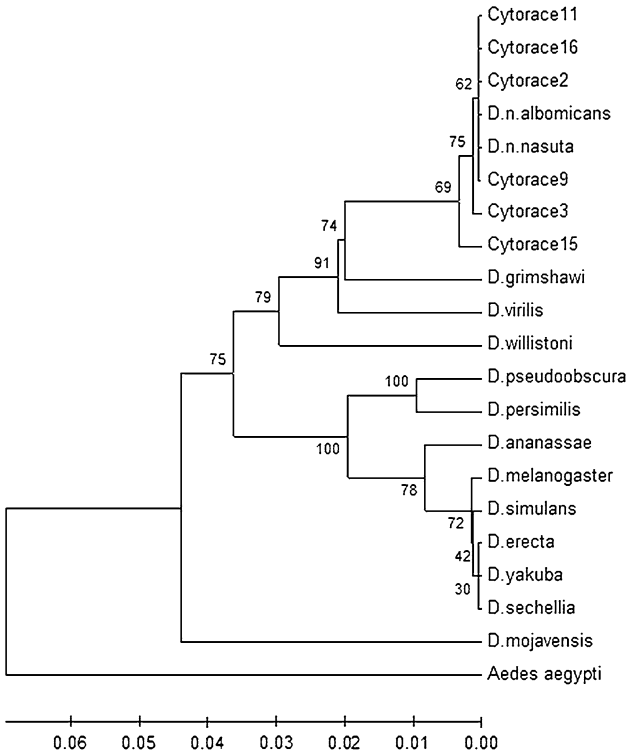

Fig. 5 The evolutionary distances were computed using the Nei-Gojobori method and are in the units of the number of aminoacid substitutions per site in *Rpd3* per sequence. The evolutionary history was inferred using the Neighbor–Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA4

acquired 6 and 5 synonymous substitutions at coding region when compared to *D. n. nasuta* and *D. n. albomicans*. The codon usage differences are more between *D. n. nasuta*, *D. n. albomicans* and members of NAC of *Drosophila* with 4, 8 and 1 % of ENC, CBI and G+C3s respectively, when compared to *D. melanogaster* and *D. simulans*.

On the other hand, the members of NAC of *Drosophila* have shown more substitutions at intron region and race specific deletions and insertions indicating the rapid divergence of intron within ~25 years as compared to *D. melanogaster* and *D. similans*. As per rough estimation, it is 4.78 synonymous mutations per MY which have occurred between the *D. melanogaster* and *D. simulans*, while it is 6.5 synonymous mutations within ~25 years in the Cytoraces. With respect to *Rpd3*, *D. melanogaser* and *D. simulans* have shown only 1–2 aminoacid changes with ~2.3 MY of divergence, while, within the span of ~25 years 1, 2, and 3 aminoacid changes are observed in C3, C16, and C15 respectively which is striking evident for rapid evolution.

Table 6 Mean concentration ± Standard error of Rpd3 and Sir2 protein in C3, C9, C15, and C16 of *nasuta-albomicans* complex of *Drosophila*

Rpd3			Sir2	
Control		Dietary restricted	Control	Dietary restricted
Cytorace 3	670 ± 32	211.5 ± 14.5	130.5 ± 14	667.6 ± 35.6
Cytorace 15	704.6 ± 18.6	213 ± 16	161 ± 17	646 ± 46
Cytorace 9	189.3 ± 14	166.2 ± 15	697 ± 20	721 ± 22
Cytorace 16	179.2 ± 12.8	159.6 ± 13.6	661 ± 24	702 ± 28

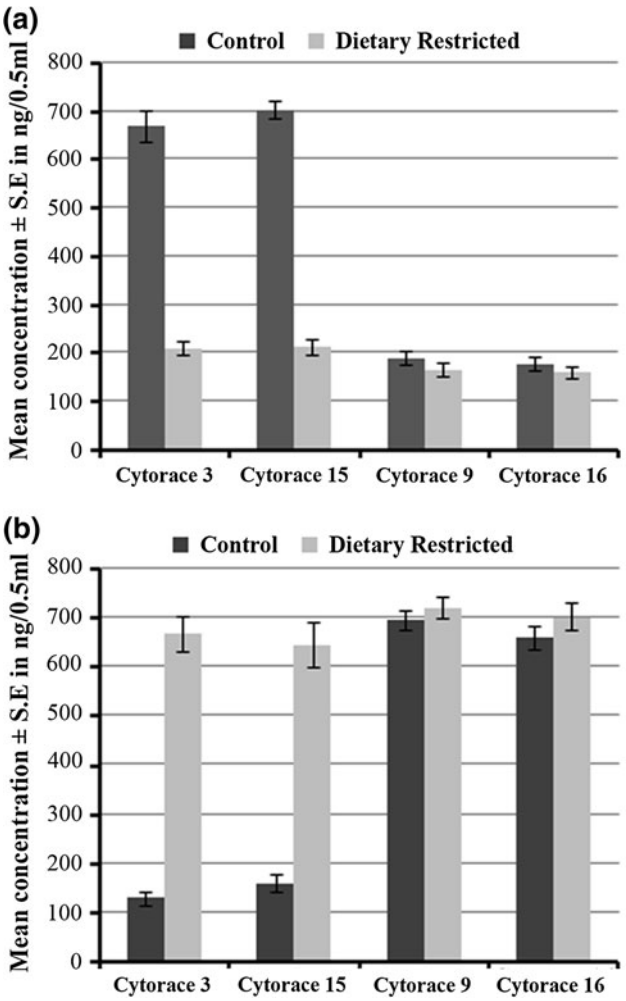

Fig. 6 Mean ± S.E concentration of Rpd3 (**a**) and Sir2 (**b**) protein in two short-lived Cytoraces, 3, 15 and long-lived Cytoraces, 9 and 16 of NAC of *Drosophila* quantified through Enzyme-Linked Immunosorbent Assay (ELISA)

Interestingly, as indicated by Bachtrog (2006), the most likely estimate of divergence time between *D. albomicans* and *D. nasuta* is about <0.5 MY. In both the genes understudy, no single nonsynonmous substitutions are observed between them. However, the hybrid cytoraces evolved by them have shown specific nonsynonymous substitutions and aminoacid change which is de novo in the context of molecular evolution.

According to Parmley and Hurst (2007) it is seductive to think that, a point mutation in a protein-coding exon that changes the DNA but not the protein sequence, would have no discernible fitness consequences. Indeed, even a decade ago such an assumption looked relatively sound. Since then, however, there has been a plethora of evidence to indicate that synonymous mutations can, indeed, have important fitness consequences, with over 40 genetic diseases now associated with such ''silent'' mutations (Chamary et al., 2006). Similarly, in the karyotypically evolved Cytoraces, the more number of synonymous mutations might be playing a role in fitness of Cytoraces.

These results are consistent with the hypothesis that a significant fraction of divergence at nonsynonymous and all noncoding sites has been driven to fixation by positive selection, and is in agreement with Andolfatto (2005). This could also be the reason where the prominent aminoacid substitutions in C15 and C16 have different effects on their lifespan.

Role of Rpd3 and Sir2 in the Regulation of Lifespan

Sirtuin 2 (Sir2) was the first gene to be found in budding yeast whose homolog in mammals is known as SIRT1 (SIR2L1 or Sir2α) and, since then, members of this highly conserved family have been found in nearly all organisms studied (Frye 2000). Sirtuins are hypothesized to play a key role in an organism's response to stresses and to be responsible for the lifespan-extending effects of calorie restriction (Sinclair and Guarente 2006). Reduction of *Rpd3* expression increases RNA levels of the histone deacetylase, *Sir2*, the direct overexpression extends lifespan (Rogina and Helfand 2004). In this study, the levels of *Rpd3* which is higher in the control with standard diet has reduced in DR treated flies of C3 and 15 and the *Sir2* levels has increased in them in DR treatment as compared to control. This implicates that higher *Rpd3* levels in C3 and C15 is one of the main cause for their shorter lifespan. In contrast, low *Rpd3* levels and no change in the *Sir2* levels of long-lived C2 and C9 showed that they do not require additional stimuli from DR to increase *Sir2* protein as it is already in adequate level in them for extended longevity. In addition to this, short-lived Cytoraces showed lower

concentration of the endogenous levels of Sod than long-lived Cytoraces (Ranjini et al. 2011).

Thus, the members of NAC of *Drosophila* offer a unique opportunity to understand both evolutionary and molecular dynamics of aging, since they are the hybrid recombination products. The Cytoraces have exhibited not only specific changes in *Sod1* and *Rpd3*, but also pronounced in the levels of synthesis of these proteins, which indicates rapid evolution of these Cytoraces in the laboratory. Further, these Cytoraces become a model system to understand the process of anagenesis.

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