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Intra- and interspecific divergence in the nuclear sequences of the clock gene *period* in species of the *Drosophila buzzatii* cluster

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Abstract

3 We have measured nucleotide variation in the CCID domain of the clock X-linked gene *period* in seven species belonging to the *Drosophila buzzatii* cluster, namely *D. buzzatii*, *Drosophila koepferae*, *Drosophila antonietae*, *Drosophila serido*, *Drosophila gouveai*, *Drosophila seriema* and *Drosophila borborema*. We detected that the purifying selection is the main force driving the sequence evolution in *period*, in agreement with the important role of CCID in clock machinery. Our survey revealed that *period* provides valuable phylogenetic information that allowed to resolve phylogenetic relationships among *D. gouveai*, *D. borborema* and *D. seriema*, which composed a polytomic clade in preliminary studies. The analysis of patterns of intraspecific variation revealed two different lineages of *period* in *D. koepferae*, probably reflecting introgressive hybridization from *D. buzzatii*, in concordance with previous molecular data.

Key words: clock genes – *period* gene – phylogeny – *Drosophila repleta* group – *D. buzzatii* cluster

Introduction

The nuclear gene *period* is an essential component of insects' circadian rhythm (Konopka and Benzer 1971; Panda et al. 2002) and also affects the courtship songs of *Drosophila melanogaster* Sturtevant (1927) males (Kyriacou and Hall 1980). Courtship songs are species specific and are one of the signals used by females to recognize conspecific males. Because *period* influences this signal, it has been considered as a potential speciation gene (Colot et al. 1988; Coyne 1992). In agreement with this idea, *period* also controls the species-specific circadian mating system in *D. melanogaster* and *D. simulans* Sturtevant (1919) (Sakai and Ishida 2001) and is linked to other features related to reproductive success in *D. melanogaster* including fecundity (Beaver et al. 2003) and copulation duration (Beaver and Giebultowicz 2004).

The *period* gene is X-linked in *Drosophila* species and composed of eight exons (Fig. 1) encoding a protein (PER) of around 1200 amino acids (Citri et al. 1987). The gene is a patchwork of relatively conserved regions surrounded by rapidly evolving regions that, in general, cannot be aligned among representatives of different species groups (Colot et al. 1988; Kyriacou et al. 1996; Tauber and Kyriacou 2008). This feature makes *period* a useful tool for comparative analyses at both intra- and interspecific levels. Indeed, this gene has been used in several comparative studies, including the *D. melanogaster* and *D. athabasca* Sturtevant and Dobzhansky (1936) complexes (Kliman and Hey 1993; Kyriacou et al. 2007) and the *D. virilis* Sturtevant (1916) and *D. willistoni* Sturtevant (1916) groups (Hilton and Hey 1996; Gleason and Powell 1997). Furthermore, homologs of *period* have been described in other insects (Barr et al. 2005; Mazzotta et al. 2005; Mazzoni et al. 2006; Regier et al. 2008), blue-green algae (Williams 2007), plants (McClung 2006) and vertebrates (Reppert and Weaver 2002). In these taxa, the *period* homologs have been successfully used to test phylogenetic (Barr et al. 2005; Regier et al. 2008) and population divergence hypotheses (Bauzer et al. 2002).

In *Drosophila*, the most studied regions of *period* are the C2 and N2 regions (Fig. 1). The former is a highly conserved region that contains the PAS domain, responsible for the 4 interaction of PER with TIM, a protein encoded by *timeless* (another important clock gene) (Panda et al. 2002; Tauber and Kyriacou 2008). The N2 region has tandemly arranged threonine–glycine residues and it has been extensively studied because of its high level of variation and its relationship with temperature compensation (Sawyer et al. 1997). Located in the C-terminal region of exon 5 is the so-called CCID domain, which contains an important component of clock machinery responsible for the transcriptional inhibitory activity of the *Drosophila* CLOCK/CYCLE heterodimer (Chang and Reppert 2003). Although there are few comparative studies using this region, it includes conserved and non-conserved stretches, allowing it to be used as a molecular marker at different taxonomic and divergence levels (Barr et al. 2005).

The *Drosophila buzzatii* cluster (*D. repleta* group, *D. buzzatii* complex) is a monophyletic group composed of seven sibling species that uses necrotic cacti tissue as breeding sites and that inhabit the open areas of sub-Amazonian regions of South America: *D. buzzatii* Patterson and Wheeler (1942), *D. koepferae* Fontdevila et al. (1988), *Drosophila antonietae* Tidon-Sklorz and Sene (2001), *Drosophila serido* Vilela and Sene (1977), *Drosophila gouveai* Tidon-Sklorz and Sene (2001), *Drosophila seriema* Tidon-Sklorz and Sene (1995) and *Drosophila borborema* Vilela and Sene (1977) (Manfrin and Sene 2006). These species have been considered as excellent models

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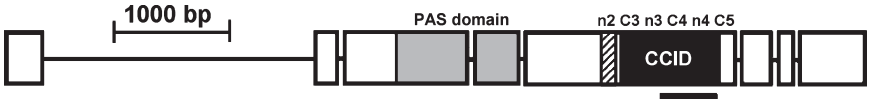

Fig. 1. Structure of *period* locus in *Drosophila melanogaster*. Introns are represented by lines and exons by boxes. The PAS domain (C2 region) is represented in grey for exons three and four. In exon five, the non-conserved regions n2, n3 and n4 are indicated, interspaced by highly conserved regions C3, C4 and C5 (Colot et al. 1988). The striped box corresponds to the threonine–glycine repetitive region (n2), and the black box represents the CCID inhibitory domain (C3 up to C5 region) (Chang et al. 2003). Under the gene structure, the black bar indicates the region analysed in the species of *D. buzzatii* cluster

for evolutionary studies mainly because of their ecological restriction with Cactaceae family, which presents a scattered distribution in South America (Sene et al. 1988; Hasson et al. 1992; Manfrin and Sene 2006). Despite the large amount of morphological, genetic and ecological data collected on these species (reviewed in Manfrin and Sene 2006), the details of the intra-cluster evolutionary relationships remain unresolved.

The phylogenetic hypothesis based on the COI mitochondrial gene suggests three evolutionary lineages for the *D. buzzatii* cluster species: one composed by *D. buzzatii* and *D. koepferae*, a second monospecific lineage that includes *D. antonietae*, and an unresolved clade that consists of *D. gouveai*, *D. borborema*, *D. seriema* and *D. serido* (Manfrin et al. 2001). In disagreement with this COI-based phylogeny, *D. antonietae* and *D. serido* share inversion $2x^7$, whereas *D. gouveai, D. seriema* and *D. borborema* share $2e^8$, a different fixed inversion, suggesting common ancestry (Ruiz and Wasserman 1993).

Based on nuclear gene sequences from the Xdh-locus, a phylogeny has been proposed for the species from the *D. repleta* Wollaston (1858) group (Rodriguez-Trelles et al. 2000). Considering the *D. buzzatii* cluster species, the main discordance between this phylogeny and those based on mitochondrial genes is the phylogenetic position of *D. koepferae*, which is more closely related with *D. serido* and *D. borborema* than with *D. buzzatii*. The absence of *D. antonietae*, *D. seriema* and *D. gouveai* from the phylogeny proposed by Rofriguez-Trelles et al. (2000) prevents a meaningful comparison with the COI phylogeny (Manfrin et al. 2001).

The contrasting phylogenetic patterns found when different markers are employed to compare the species of the *D. buzzatii* cluster appear to be a consequence of the intrinsic difficulties associated with reconstructing the phylogenetic history of closely related species, such as recent divergence time and introgression (Machado and Hey 2003). The species from the *D. buzzatii* cluster present a complex pattern of population differentiation, apparently diverging in a mosaic fashion (Sene et al. 1988) with the occurrence of introgressive hybridization between different evolutionary lineages within the cluster (Manfrin et al. 2001; de Brito et al. 2002; Gómes and Hasson 2003; Piccinali et al. 2004).

Three main objectives are pursued in this study. First, we aim to characterize a fragment of the *period* CCDI domain (Fig. 1) and introduce its use as molecular marker in the *D. buzzatii* cluster species. Second, we aim to unravel phylogenetic relationships within the *D. buzzatii* cluster. Thirdly, we aim to assess the extent of introgressive hybridization in the entire cluster, reported in certain species pairs by means of surveys of sequence variation in mitochondrial DNA (Manfrin et al. 2001) and nuclear genes (Gómez and Hasson 2003; Piccinali et al. 2004). We choose *period* as molecular marker because the species of *D. buzzatii* cluster have different courtship songs (Oliveira 2008), making this gene a candidate for evolutionary changes in this species group.

Material and Methods

Samples

A total of eighty-seven individuals from all species of the *Drosophila buzzatii* cluster were collected from different populations of each species and analysed. The sample details are presented in Table 1.

Isolation of period gene fragments

The genomic DNA of adult male flies was extracted with Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA). First, we amplified fragments of the *period* gene region using degenerate primers per2887 and per3887 (designed in Prof. Reinaldo de Brito's research group – UFSCAR). The conditions of PCR were the same as in Gleason and Powell (1997). The fragments isolated in this initial step (around 550 bp) were eluted from 1% agarose gel using the GFX PCR DNA and Gel Band Purification kit' (GE Healthcare, Buckinghamshire, UK). The recovered fragments were cloned using the pMOSBlue blunt-ended cloning kit RPN 5110 (GE Healthcare) to design specific primers. Recombinant clones were identified using the β-galactosidase blue–white selection system (Sambrook et al. 1999). Plasmid DNA was prepared according the methodology described in Sambrook et al. (1999), and the DNA template reaction for sequencing was prepared according to the BigDye Terminator Cycle Sequencing Ready Reaction kit manual (Perkin-Elmer, Foster City, CA, USA), using the universal primer M13. Automatic DNA sequencing was performed on an ABI Prism™ 377 sequencer. The sequences obtained were then utilized to design two specific primers for the *D. buzzatii* cluster: perCBf (5′ TGGGAGGGCGAGGCGAACAA 3′) and perCBr (5′ GGCATGGGTTGGTACATCAT 3′) (Fig. 1), allowing better yields in PCR amplifications.

The perCBf and perCBr primers were utilized to both amplify and sequence fragments of the *period* gene in all samples listed on Table 1. As the *period* locus is X-linked in species of *Drosophila* of the subgenus *Sophophora*, directly sequencing was possible using exclusively male individuals in our sample. This methodological advantage is possible because males have only one copy of the X-linked gene. The absence of overlapping peaks in the chromatograms generated by sequencing suggests that the *period* locus is also found on the X chromosome in the species of the *D. buzzatii* cluster species. The purification and sequencing procedures were performed as described earlier.

Data analyses

The alignment of the sequences was carried out using the CLUSTAL W program v1.8 (Thompson et al. 1994) and edited in BioEdit (Hall 1999). Sequence homology searches were performed on the NCBI GenBank using BLASTn (Altschul et al. 1990). Standard indices of nucleotide diversity (π), number of polymorphic sites (S), number of synonymous and non-synonymous changes were computed using DNAsp 4.20 (Rozas et al. 2003), and the number of shared polymorphism and fixed differences between groups were computed using SITES software (Hey and Wakeley 1997). We defined shared polymorphism and fixed differences following the tutorial of SITES: fixed difference is a polymorphic site where all the sequences of one group are different from all of the sequences of a second group; and shared polymorphism is a polymorphic site where two groups shared at least two segregating nucleotides. To detect recombination, we calculated the *Rm* parameter, which estimates the minimum number of recombination events in a DNA sample (Hudson and Kaplan 1985).

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Table 1. Samples used in the study

Species	Location	LC	N	Geographical Coordinates	Access Number
Drosophila buzzatii	Serra do Cipó, Brazil	N57	4	19.3º S, 43.6º W	FJ267303-FJ267306
	Grão Mogol, Brazil	D54	1	16.6º S, 42.9º W	FJ267312
	Petrolina, Brazil	N36	5	9.1º S, 40.6º W	FJ267307-FJ267311
	San Juan, Argentine	H98	1	31.58 S, 68.52 W	FJ267302
Drosophila koepferae	Famatina, Argentine	B26D2	1	29.1º S, 67.2º W	FJ267313
	Suyuque, Argentine	SuyDK	5	33.3º S, 66.5º W	FJ267314-FJ267318
Drosophila antonietae	Santiago, Brazil	J27	4	29.2º S, 54.8º W	FJ267319-FJ267322
	Serrana, Brazil	J38	4	21.3º S, 47.6º W	FJ267323-FJ267326
	Cianorte, Brazil	D93	1	23.7º S, 52.6º W	FJ267327
Drosophila serido	Milagres, Brazil	J92/1431	5	11.2º S, 39.9º W	FJ267328-FJ267332
	Arraial do Cabo, Brazil	N20	5	23.0º S, 42.0º W	FJ267333-FJ267337
	Bertioga, Brazil	H49	1	23.9º S, 46.1º W	FJ267338
	Mucugê, Brazil	N45	1	13.0º S, 41.4º W	FJ267339
	Morro do Chapéu, Brazil	N39	1	11.6º S, 41.2º W	FJ267340
Drosophila gouveai	Ibotirama, Brazil	J78	5	12.1º S, 43.3º W	FJ267341-FJ267345
	Juazeiro, Brazil	N47	4	9.5º S, 40.5º W	FJ267361-FJ267364
	Petrolina, Brazil	N36	5	9.1º S, 40.6º W	FJ267356-FJ267360
	Cristalina, Brazil	J75	5	16.7º S, 47.7º W	FJ267346-FJ267350
	Altinópolis, Brazil	H6G6	1	21.1º S, 47.6º W	FJ267355
	Analândia, Brazil	J67	4	22.1º S, 47.7º W	FJ267351-FJ267354
Drosophila borborema	Milagres, Brazil	J92	5	11.2º S 39.9º W	FJ267365-FJ267369
	Morro da Barrinha, Brazil	N37	5	9.9º S, 40.3º W	FJ267370-FJ267374
	Juazeiro, Brazil	N47	2	9.5º S, 40.5º W	FJ267375-FJ267376
	Grão Mogol, Brazil	N48	4	16.6º S, 42.9º W	FJ267377-FJ267380
Drosophila seriema	Morro do Chapéu, Brazil	N39	2	11.6º S, 41.2º W	FJ267381-FJ267382
	Mucugê, Brazil	N45	5	13.0º S, 41.4º W	FJ267383-FJ267387
	Serra do Cipó, Brazil	D40F1	1	19.3º S, 43.6º W	FJ267388

LC, Location code; N, Number of individuals.

The MODELTEST 3.7 program (Posada and Crandall 1998) was used to determine the model of evolution using the Akaike information criterion (AIC). The HKY+I+G (Hasegawa et al. 1985), with gamma shape = 1.0264 and pinvar = 0.5078, was the best-fit model of nucleotide substitution selected for the *period* gene sequences. When performing saturation tests in the program DAMBE 5.0 10 (Xia and Xie 2001), however, we used the Tamura-Nei model (Tamura and Nei 1993), which contains the HKY+I+G premises (Schneider 2007), as DAMBE does not have the HKY+I+G model in its data base.

The distance matrix was used to create a phenogram using the neighbour-joining (NJ) method (Saitou and Nei 1987). Genetic distance calculations and phenograms were made using MEGA 4.0 (Tamura et al. 2007). Additional phylogenetic analyses were performed using maximum parsimony (MP) and maximum likelihood (ML) optimization criterions using the PAUP*4.0b10 program (Swofford 2002), with *D. mojavensis* Patterson and Crow (1940) and *D. hydei* Sturtevant (1921) as outgroups (GenBank accession FJ267300 and FJ267301, respectively). Heuristic searches were performed using parsimony (Nixon 1999) and likelihood (Morrison 2007) ratchet in conjunction with PRAP 2.0b3 program (Müller 2004), to prepare commands to be executed by PAUP*. The settings for the parsimony ratchet were as follows: ratchet replicates = 1000, random addition cycles = 10, weight = 2, % weighted = 25. All characters were weighted equally. Node support was assessed using bootstrap proportions (Felsenstein 1985) with 5000 pseudo-replicates and Bremer support (Bremer 1994). Decay values were also calculated using the PRAP 2.0b3 program (Müller 2004). ML trees were inferred using a neighbour-joining starting tree and a subtree-pruning-regrafting (SPR) algorithm for branch swapping. For ML analysis the likelihood ratchet was implemented using 200 ratchet replicates, weight = 2, % weighted = 25. A strict consensus tree with the same likelihood was considered the best estimate of the gene phylogeny. Bootstrap frequencies for the ML were calculated using the program PhyML (Guindon and Gascuel 2003) with 500 pseudo-replicates. The TREEGRAPH 1.0 rc3 program (Müller and Müller 2004) was used to draw the phylogenies.

To detect adaptive evolution, the relative rates of synonymous (ds) and non-synonymous (dn) substitutions were determined according to the Nei and Gojobori method using the Jukes and Cantor correction (Nei and Gojobori 1986). The significance of the difference between dn and ds rates was tested using a Z-test of selection at the 5% level, whereby the p values are the probability of rejecting the null hypothesis of neutrality (H_0: dn = ds, Nei and Kumar 2000). MEGA 4.0 (Tamura et al. 2007) was used to perform the Z-test. Site- and branch-specific selection was studied on the Selecton server (available at http://selecton.bioinfo.tau.ac.il). Selecton calculates ω for each codon position using a Bayesian inference approach. To detect positive selection, the ML phylogenetic tree produced was used as input in Selecton and the M8 evolutionary model (Yang et al. 2000) was compared with M8a (Swanson et al. 2003) and M7 (Yang et al. 2000) models, by means of a likelihood ratio test (LRT), as suggested in the study by Stern et al. (2007).

Results

Characterization and polymorphism of the isolated period fragments

A total of 443 bp of the *period* gene were obtained from 87 individuals belonging to seven species of the *Drosophila buzzatii* cluster (Table 1). The sequences contain the conserved regions C4 and C5, interspaced by non-conserved regions N3 and N4, within the CCID domain (Colot et al. 1988; Chang and Reppert 2003; Fig. 1). An alignment of the deduced amino acid sequences of *Drosophila buzzatii* and *D. mojavensis* with the PER A protein described for *D. melanogaster* and allied species showed a high degree of conservation of regions C4 and C5 (Fig. 2).

The isolated *period* sequences are CG-rich (~60% on average) especially in the third codon positions (CG content in the coding region: first codon position: 55%, second: 56.5%, third: 71.5%). Table 2 presents standard measures of intraspecific variation. Estimates of synonymous variation (πs) were always higher than replacement variation (πr) in all species

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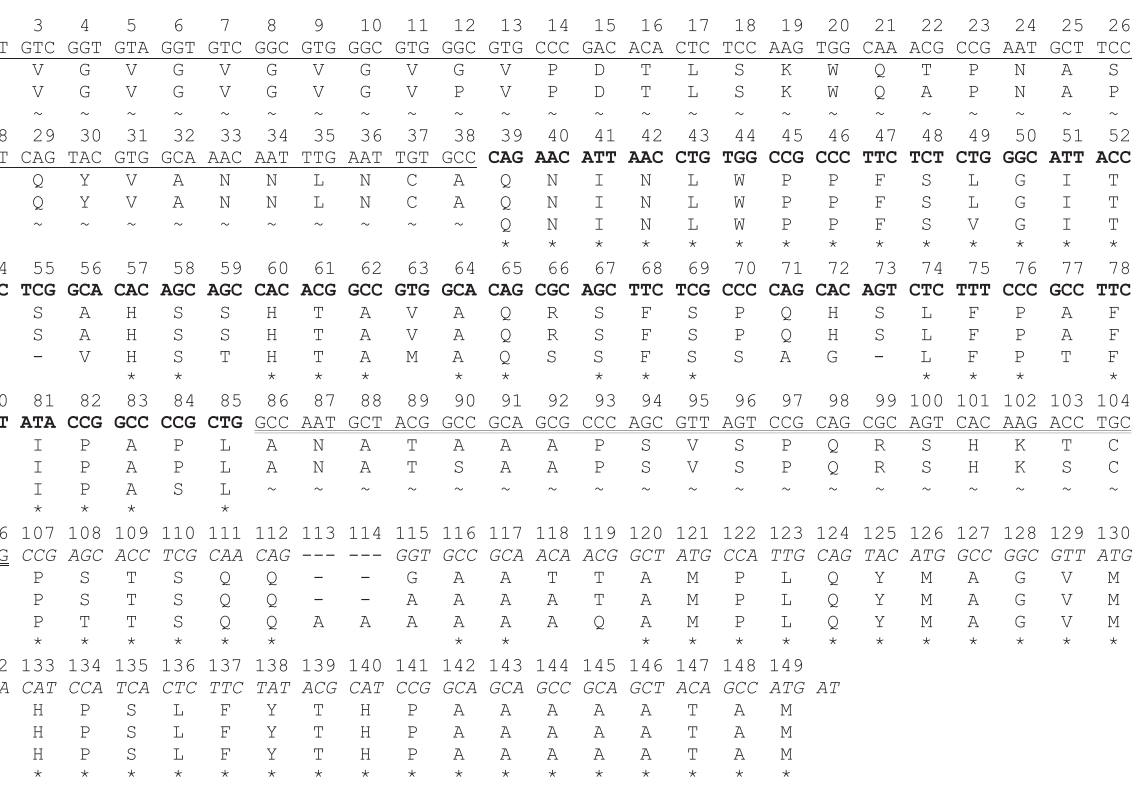

Fig. 2. Alignment containing the deduced amino acid sequences of the C-terminal region of the *period* gene of *Drosophila buzzatii* (DbuzE3) and *D. mojavensis* (Dmoj) species, with the amino acid sequences of *D. melanogaster* (Dmel) PER A protein (Access number NP_525056). Above the amino acid alignment is the nucleotide sequence of *D. buzzatii* species. The underline and double underline sequences correspond to n3 and n4 non-conserved regions, respectively. The bold and italic sequences refer, respectively, to C4 and C5 conserved regions. (*) indicates similar amino acids shared among the three species. (∼) indicates regions where the alignment of *Drosophila buzzatii* and *D. mojavensis* with *D. melanogaster* sequences was not possible. (−) indicates indels

Table 2. Xxxxxxxxxxxxx

investigated (Table S1). Except for *D. antonietae*, all species showed evidence of at least one intragenic recombination event.

The highest scores of nucleotide diversity were obtained in *D. koepferae* (Table S1). However, it is worth noting that two distinct evolutionary lineages appear to be present in this species: koep1, represented by the alleles B26D2 and SuyDK41, and koep2, represented by of the remaining four sequences. Interestingly, the latter appears to be more related to the *D. buzzatii* alleles than to koep1 (Figs 3 and 4). The coexistence of two relatively well-differentiated lineages in *D. koepferae* could explain the high values of nucleotide diversity recorded in *D. koepferae* species (Table S1).

Except for the comparison between *D. buzzatii* and koep2, which did not have fixed nucleotide differences, pairwise comparisons revealed at least one fixed difference in pairwise comparisons between species (Table S2). Estimates of genetic distances between species (Table S2) indicate that *D. buzzatii* is the most differentiated species relative to the other species of the *D. buzzatii* cluster whereas *D. borborema* and *D. seriema* are the most similar species (Fig. 3).

The shared nucleotide polymorphisms were detected in our survey at the intra- and interspecific level in the *D. buzzatii* cluster. *D. buzzatii* and koep2 were the entities with the largest number of shared nucleotide polymorphisms (Table S2). Moreover, *D. serido* shared polymorphic variants at three nucleotide sites with both *D. buzzatii* and *D. koepferae*, and *D. borborema* shared three polymorphisms with *D. seriema*. In the additional pair-to-pair comparisons, 0–1 shared polymorphisms were found (Table S2).

Phylogenetic analyses

The multiple alignment of the 89 sequences of the 443 bp of *period* gene displayed 123 variable sites, of which 72 were parsimoniously informative. Saturation tests revealed that in most cases both transitions and transversions remained informative. However, a portion of the curve for transitions appears to become partially saturated above the 12% divergence value (Figure S1), indicating the occurrence of multiple substitutions and homoplasy in this mutation class.

The MP (not shown) and the ML tree (Fig. 4) presented very similar topologies, differing only in the bootstrap values in the main branches (Fig. 4). The both trees are also similar to the NJ phenogram (Fig. 3). The main results of our phylogenetic analyses are as follows. First, we confirm the monophyletic status of the *Drosophila buzzatii* cluster. Second, two fairly well differentiated lineages were observed in *D. koepferae*. One of the lineages (koep2) was grouped together with the *D. buzzatii* sequences, in a clade supported by high bootstrap values (Figs 3 and 4), whereas the koep1 lineage was allocated with *D. buzzatii* + *D. koepferae* koep2 only in the ML tree,

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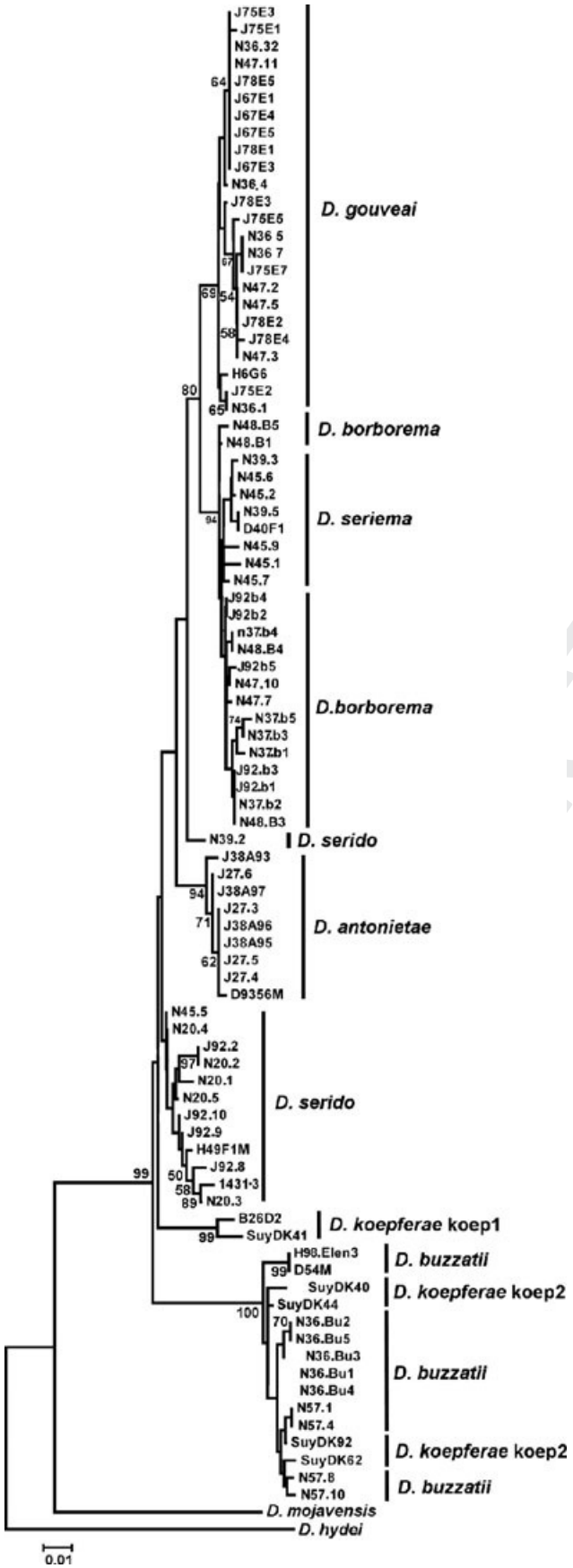

but with low bootstrap values (Fig. 4). In the MP tree (not shown), the sequences that compose the *D. koepferae* koep1 lineage were allocated in a distinct clade, and the genetic distance data indicate that this lineage is more similar to the remaining species of the *D. buzzatii* cluster than to *D. koepferae* koep 2 and *D. buzzatii* (Table 2, Fig. 3). Third, *D. antonietae* and *D. serido* formed two monophyletic groups, with high and low statistical support, respectively (Figs 3 and 4). Fourth, *D. gouveai*, *D. borborema* and *D. seriema* are grouped in one clade with high bootstrap values (Figs 3 and 4). Fifth, *D. borborema* and *D seriema* composed a clade, but with unresolved relationships (Figs 3 and 4, Table S2).

Tests of the neutral model

The Z-test of natural selection, based on the Nei and Gojobori method (Nei and Gojobori 1986), suggest that *period* CCID domain is under strong purifying selection (Table 3). To confirm these results, the null hypothesis of neutral evolution was also tested using a Bayesian approach (Stern et al. 2007). Using the M8 model (Yang et al. 2000), we detected a dn/ds ratio significantly greater than one ($\omega = 1.4$), supposedly an indication of positive selection, in only one amino acid site (position 115 in Fig. 2). However, the lower bound of the 95% confidence interval (0.12–4.9) of the ω computed for this site was lower than one, contradicting the suggestion of positive selection (Stern et al. 2007). Moreover, when the results of the M8 model simulation were compared with models M8a (Swanson et al. 2003) and M7 (Yang et al. 2000) by means of LRT, the positive selection signal was not statistically significant. The remaining amino acid positions presented a ω value lower than 1 with a 95% confidence interval not including 1, which may be interpreted as purifying selection, in general agreement with the results of the Z-test (Table S3).

Discussion

The studies concerning the *period* gene are biased taxonomically within the *Drosophila* genus: large amounts of data are available for flies of the subgenus *Sophophora*, but little is known of the subgenus *Drosophila*, especially within the *D. repleta* species group. This group is among the largest of all groups in the genus *Drosophila*, containing more than 90 species, naturally endemic to the Neotropical region (Vilela 1983; Durando et al. 2000). Here, we show that *period* may be considered as a useful tool for studying inter- and intraspecific variation in the *D. buzzatii* cluster of the *D. repleta* species group. The inclusion of all species of the *D. buzzatii* cluster in this study allows us to identify an important number of informative nucleotide variants (fixed differences), indicating that *period* is an effective marker for species identification in this cluster, which is composed of cryptic species that are sympatric in several locations of their geographical range (Manfrin and Sene 2006).

The alignment of the C4 and C5 regions of *period* sequences of *D. buzzatii* and *D. mojavensis* (*D. repleta* group) and

Fig. 3. A neighbour-joining phenogram showing the relationships (genetic distances) of the *period* gene fragments analysed in this work. Numbers above branches indicate bootstrap values (> 50%). The scale bar represents genetic distances calculated according to Tamura and Nei model (Tamura and Nei 1993)

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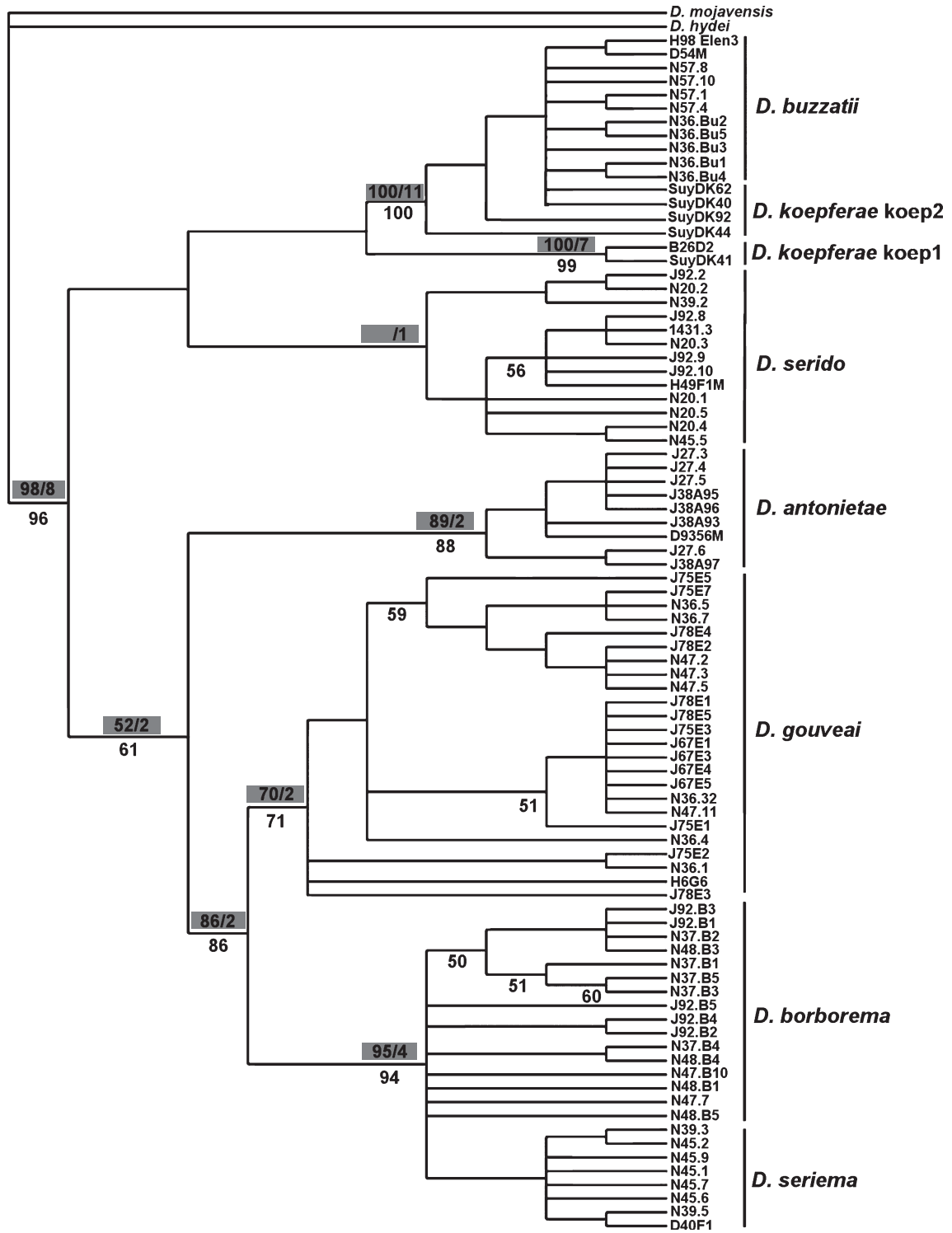

Fig. 4. Maximum likelihood (ML) tree obtained using the HKI + I + G model (Hasegawa et al. 1985), as proposed by the Akaike information criterion (AIC) test. Numbers below branches indicate bootstrap values (> 50%) of the ML analysis. Branch lengths are not proportional to the amount of change. In the grey boxes above branches the numbers indicate statistical support of the main clades obtained in maximum parsimony analysis (72 parsimony-informative characters, 207 steps, CI = 0.729, RI = 0.936): bootstrap values/Bremer support

D. melanogaster revealed a high degree of sequence conservation, despite the long divergence time (around 40 million years ago) between the subgenera *Drosophila* and *Sophophora* to which these species belong (Markow and O'Grady 2007). This high degree of sequence conservation among species that diverged for long evolutionary periods, and even among insects belonging to distantly related groups, such as *Ceratitis capitata* Wiedemann (1824) (Mazzotta et al. 2005), indicates a primordial role for the C4 and C5 regions. Indeed, these regions are included in the CCID domain that plays an important role in circadian clock regulation: the transcriptional inhibitory activity of the *Drosophila* CLOCK/CYCLE

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Table 3. Xxxxxxxxxxxxx

heterodimer (Chang and Reppert 2003), which in turn binds to *period* and *timeless* promoters, stimulating transcription (Tauber and Kyriacou 2008). In general, agreement with this important role in clock machinery, the fragment of *period* analysed is under purifying selection (Table S2).

The phylogenetic position of *D. koepferae* within the *D. buzzatii* cluster has been a controversial issue. Based on mitochondrial DNA sequence data, *D. koepferae* and *D. buzzatii* appear as sister taxa. Studies based on nuclear markers (Rodriguez-Trelles et al. 2000; Franco et al. 2008a) and aedeagus and wing morphology (Moraes and Sene 2007; Manfrin and Sene 2006) suggest that *D. koepferae* is more closely related to the so-called *serido* sibling set than to *D. buzzatii*. These contrasting patterns could be explained as a result of shared polymorphisms between the *D. koepferae* and *D. buzzatii* genomes, such as the shared polymorphism in the *period* gene, as observed in this study (Table S2).

There are at least two alternative explanations, non-mutually exclusive, to explain the shared polymorphic variants between species: maintenance of ancestral polymorphism and/or introgressive hybridization (Hey 2006). To explain the high similarity between the *D. koepferae* koep2 lineage and the *D. buzzatii* species, the former possibility seems to be unlikely, as these species diverged around 4 Myr ago (Gómes and Hasson 2003; Manfrin and Sene 2006), more than enough time to lose most shared polymorphisms (Clark 1997). In this sense, the detection of two distinct evolutionary lineages in *D. koepferae* (koep1 and koep2), but not in *D. buzzatii*, suggests unidirectional introgression, probably as the outcome of backcrosses of fertile F1 hybrid females with *D. koepferae* males, because hybrid males are sterile (Soto et al. 2008). Giving support to this hypothesis, these species are sympatric in many locations of the Chaco Domain, and laboratory experiments indicate that these species are capable of gene exchange (Machado et al. 2006; Soto et al. 2008). Moreover, previous studies based on two nuclear genes also suggest introgressive hybridization between *D. buzzatii* and *D. koepferae* (Gómez and Hasson 2003; Piccinali et al. 2004).

The locality of Suyuque, where we found the distinct *period* lineages in *D. koepferae* (Figs 3 and 4), is an area where *D. buzzatii* and *D. koepferae* coexist. Recent laboratory studies show that, in some crosses, hybrids were at least as viable and developed as fast as parental species (Soto et al. 2008), in agreement with our hypothesis of introgressive hybridization between these two species. Moreover, development was not affected by interspecific hybridization, at least as measured by departures from bilateral symmetry (Carreira et al. 2008).

Introgressive hybridization between closely related species seems to be a more common phenomenon than that previously acknowledged and appears to be widespread in animal taxa (Seehausen 2004). In the *D. buzzatii* cluster, this phenomenon does not appear to be restricted to *D. koepferae* and *D. buzzatii*. Actually, mitochondrial haplotypes characteristic of *D. antonietae* were detected in the southernmost limit of *D. gouveai*'s distribution, including the Analândia locality, probably because of secondary contact between these species as a consequence of population range expansions during the glacial periods (Manfrin et al. 2001; de Brito et al. 2002). In this work, *D. gouveai* sequences from Analândia were grouped together with the other *D. gouveai* populations in the same clade and did not share any nucleotide variant with *D. antonietae* (Figs 3 and 4), suggesting that introgressive hybridization affected mitochondrial and nuclear genes of this species differentially, as already suggested previously (Franco et al. 2006a).

Surveys of sequence variation in the *period* gene also provided evidence suggestive of introgressive hybridization in other fruit flies, such as the close relatives *D. pseudoobscura* Frolova and Astaurov (1929) and *D. persimilis* Dobzhansky and Epling (1944) (Wang and Hey 1996) and in the closely related species *Lutzomyia intermedia* Lutz and Neiva (1912) and *L. whitmani* Antunes and Coutinho (1912) (Diptera, Psychodidae) (Mazzoni et al. 2006). These events of introgression were highlighted to refute the idea of *period* as a speciation gene in these groups. Due the introgression evidences found in the present paper, this same suggestion could be also ascribed to the *period* gene fragment of the *D. buzzatii* cluster species.

In *D. antonietae*, *period* sequences formed a monophyletic group with high statistical support (Figs 3 and 4), and we found the lowest levels of interpopulation variation. This result is coincident with the great genetic homogeneity observed for the mitochondrial DNA (Manfrin et al. 2001), allozymes (Mateus and Sene 2007), satellite DNA (Franco FF and cols. unpublished data) and aedeagus morphology (Franco et al. 2006b) of this species. Extensive gene flow in this species is facilitated by its association with a widely distributed host cactus, *Cereus hildmaniannus* K. Schum, which occurs in mesophile gallery forests along the rivers of the Paraná-Paraguay basin, forming probable migration corridors for *D. antonietae* (Manfrin and Sene 2006; Mateus and Sene 2007). An alternative hypothesis to explain the low level of diversification among the populations of *D. antonietae* is a past recent bottleneck as consequence of the contraction of xerophytic vegetation cover in South America after the last glacial period (Ab' Saber 1977; Pennington et al. 2000).

The populations of *D. serido* shared the fixed $2x^7$ chromosome inversion (Ruiz et al. 2000), but this species is polytypic with regard to metaphase chromosomes (Baimai et al. 1983), frequencies of polymorphic inversions (Ruiz and Wasserman 1993; Ruiz et al. 2000), mitochondrial DNA haplotypes (Manfrin et al. 2001) and aedeagus morphology (Franco et al. 2008b). *D. serido* showed the highest levels of nucleotide diversity in the *period* gene, and its sequences were grouped in a branch with low support in the phylogenetic analyses (Figs 3 and 4), in agreement with its status as a polytypic species (Manfrin and Sene 2006).

The phylogenetic position of *D. serido* could not be elucidated in the present work. In disagreement with mitochondrial DNA data, *D. serido* did not form a monophyletic group with *D. gouveai*, *D. seriema* and *D. borborema*. These results are in agreement with the cytological evolution depicted by shared fixed chromosome inversions. *D. serido* shares with *D. antonietae* the $2x^7$ inversion, while *D. gouveai*, *D. borborema* and *D. seriema* share inversion $2e^8$ (Ruiz and Wasserman 1993; Ruiz et al. 2000). This study shows that the latter three species form a monophyletic group, in which *D. gouveai* appears as the sister group of *D. seriema* and *D. borborema*, in agreement with chromosome inversion data.

D. seriema and *D. borborema* are in the same clade, but without reciprocal monophyly (Figs 3 and 4) probably because

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Table 4. Xxxxxxxxxxxxx

of the sharing of three polymorphic variants (Table 4). This fact could be consequence of introgressive hybridization because these species are sympatric in many locations. However, there is no empirical data on the degree of reproductive isolation between *D. seriema* and *D. borborema* to corroborate this hypothesis. As these species compose a relatively recent lineage within *D. buzzatii* cluster, we conjecture that the maintenance of ancestral polymorphisms is the most plausible explanation for the nucleotide variants shared between *D. seriema* and *D. borborema*.

The species of the *Drosophila buzzatii* cluster comprise a range of divergences, degrees of reproductive isolation (Machado et al. 2006; Manfrin and Sene 2006), ecological restrictions and cacti hosts (Sene et al. 1988), allowing their use as a model in different fields of evolutionary biology (Manfrin and Sene 2006), such as, for example, molecular evolution of satellite DNA families (Kuhn et al. 2007), phenotypic plasticity (Carreira et al. 2008), life history characters (Soto et al. 2008), population genetics and phylogeography (de Brito et al. 2002; Mateus and Sene 2007; Moraes and Sene 2007), and aedegus evolution (Soto et al. 2007; Franco et al. 2008b). Additionally, these species are a promising model to study, for example, cacti host shift and speciation genetics. Our molecular data provide a much-needed phylogenetic framework, consistent with that based upon chromosomal inversions, which can serve as the basis for future evolutionary studies of the *D. buzzatii* species cluster.

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Resumen

Divergencia inter y intraespecifica en las secuencias nucleares del gene period en las especies del ''cluster'' Drosophila buzzatii.

En este trabajo hemos medido la variación nucleotídica en el dominio CCID en el gen *period*, localizado en el cromosoma X, asociado a los ritmos circadianos, el enjambre de siete especies que conforman denominado ''cluster'' *D. buzzatii*: *D. buzzatii*, *Drosophila koepferae*, *Drosophila antonietae*, *Drosophila serido*, *Drosophila gouveai*, *Drosophila seriema* y *Drosophila borborema*. Nuestro estudio mostró que la selección purificadora es la principal fuerza que gobierna la evolución de *period*, en acuerdo con una región que como CCID juega un papel importante en la maquinaria de los ritmos circadianos. Hemos observado que la variación en *period* provee información filogenética que permitió resolver la politomía que involucra a *D. gouveai*, *D. borborema* y *D. seriema*. Finalmente, el análisis de la variación intraespecifica reveló la presencia de dos linajes en *D. koepferae*, uno de los cuales podría ser consecuencia de hibridación introgressiva desde *D. buzzatii*, en acuerdo con estudios anteriores en regiones génicas localizadas en otros cromosomas.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. Polymorphism statistics and minimum number of recombination events applied to *period* gene sequences of the species of *Drosophila buzzatii* cluster.

Table S2. Pairwise comparison of fixed differences and shared polymorphisms among the *Drosophila buzzatii* cluster species, where the shared polymorphism is shown in parenthesis (above diagonal). Average intraspecific (diagonal) and interspecific (below diagonal) genetic distances values were based on the Tamura and Nei model [Tamura and Nei (1993)].

Table S3. Results of Z-test of selection based on the Nei and Gojobori method (Nei and Gojobori 1986).

Figure S1. Transition (s) and transversion (v) rates plotted against divergence among sequences of the nuclear gene period based on the Tamura and Nei model (Tamura and Nei 1993).

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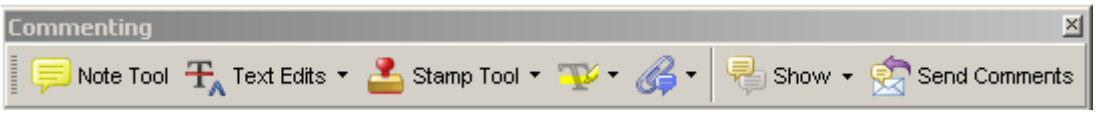

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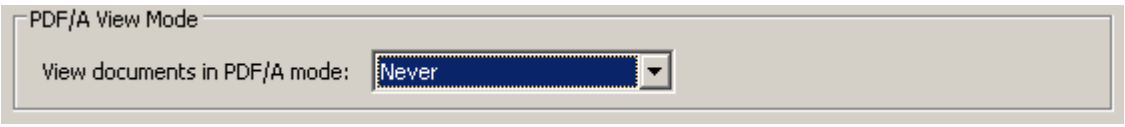

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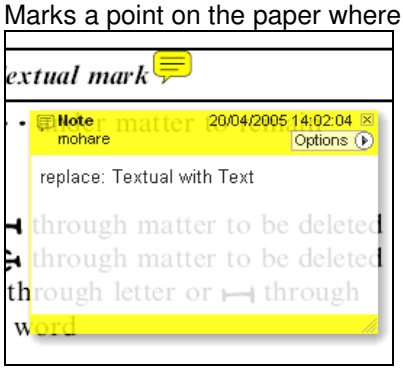

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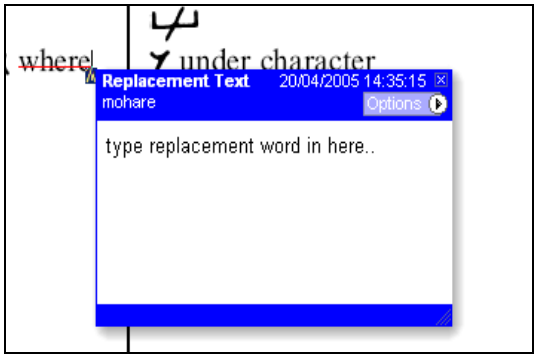
1. Right click into area of either inserted text or relevance to note
2. Select Add Note and a yellow speech bubble symbol and text box will appear
3. Type comment into the text box
4. Click the X in the top right hand corner of the note box to close.

Replacement text tool — For deleting one word/section of text and replacing it

Strikes red line through text and opens up a replacement text box.

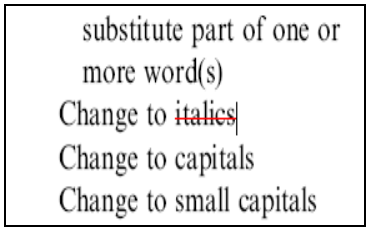


How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Replace Text (Comment) option
5. Type replacement text in blue box
6. Click outside of the blue box to close

Cross out text tool — For deleting text when there is nothing to replace selection

Strikes through text in a red line.



How to use it:

1. Select cursor from toolbar
2. Highlight word or sentence
3. Right click
4. Select Cross Out Text

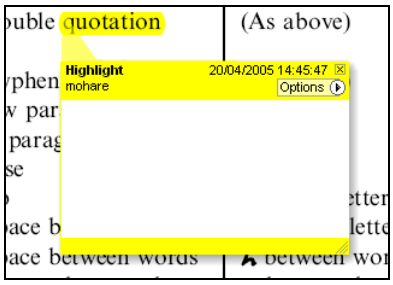
Approved tool — For approving a proof and that no corrections at all are required.

How to use it:

1. Click on the Stamp Tool in the toolbar
2. Select the Approved rubber stamp from the 'standard business' selection
3. Click on the text where you want to rubber stamp to appear (usually first page)

Highlight tool — For highlighting selection that should be changed to bold or italic.

Highlights text in yellow and opens up a text box.

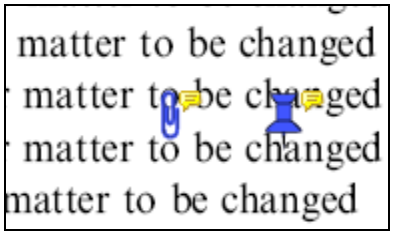


How to use it:

1. Select Highlighter Tool from the commenting toolbar
2. Highlight the desired text
3. Add a note detailing the required change

Attach File Tool — For inserting large amounts of text or replacement figures as a files.

Inserts symbol and speech bubble where a file has been inserted.

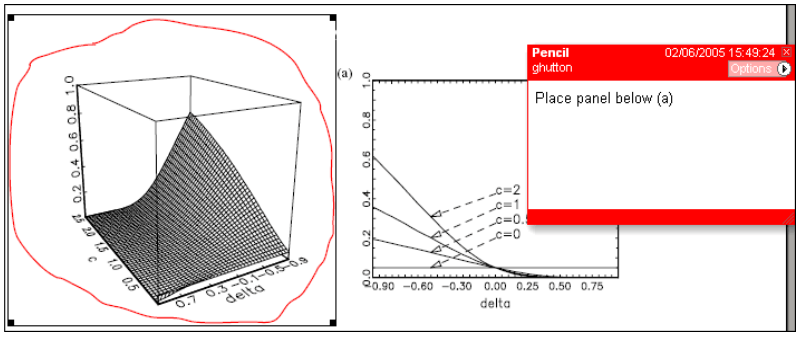


How to use it:

1. Click on paperclip icon in the commenting toolbar
2. Click where you want to insert the attachment
3. Select the saved file from your PC/network
4. Select appearance of icon (paperclip, graph, attachment or tag) and close

Pencil tool — For circling parts of figures or making freeform marks

Creates freeform shapes with a pencil tool. Particularly with graphics within the proof it may be useful to use the Drawing Markups toolbar. These tools allow you to draw circles, lines and comment on these marks.



How to use it:

1. Select Tools > Drawing Markups > Pencil Tool
2. Draw with the cursor
3. Multiple pieces of pencil annotation can be grouped together
4. Once finished, move the cursor over the shape until an arrowhead appears and right click
5. Select Open Pop-Up Note and type in a details of required change
6. Click the X in the top right hand corner of the note box to close.

Help

For further information on how to annotate proofs click on the Help button to activate a list of instructions: