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碩士論文

Institute of Zoology
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Master Thesis

黃果蠅嗅覺受器 *33c* 的分子演化

Molecular evolution of *odorant receptor 33c* in *Drosophila melanogaster*

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摘要

果蠅的表皮碳氫化合物具有性費洛蒙的功能，在物種間的識別及生殖隔離方面扮演很重要的角色。果蠅的嗅覺器官為觸角及小顎鬚，先前對多種常見氣味的測試，都未發現小顎鬚上的嗅覺受器 *33c* (*Or33c*)有任何反應，推測費洛蒙有可能是此受器有反應的未知氣味。另外，在不同物種間，*Or33c* 已被證實受到正向選擇的作用。為了更進一步了解果蠅 *Or33c* 的功能以及正向選擇對於 *Or33c* 演化的影響，我首先比較了 *Or33c* 在不同族群及性別中的表現量，發現 *Or33c* 在黃果蠅中的表現具有雌雄雙型性。另外，經由比較 *Or33c* 和其縱向複製基因 *Or33a* 及 *Or33b* (表現於觸角)的序列多型性，發現無論是在非洲族群或非非洲族群中，*Or33c* 皆受到了正向選汰的作用，且 *Or33b* 和 *Or33c* 間的基因區段亦受到此正向選汰的作用而發生 hitchhiking 現象。*Or33c* 具有一個使其能在小顎鬚表現的正向調控模段，此模段的獲得可能與其轉換到不同感受器官上表現及所受之正向選汰有關。了解何種因素驅動了 *Or33c* 的正向選汰，將能更進一步釐清 *Or33c* 在偵測表皮碳氫化合物上的可能功能。

關鍵字：表皮碳氫化合物，黃果蠅，嗅覺受器，正向選汰，雌雄雙型性

Abstract

Pheromonal cuticular hydrocarbons (CHs) have been demonstrated to play important roles in species recognition and sexual isolation in *Drosophila*. Previous study showed that *odorant receptor 33c* (*Or33c*) locating on one of the *Drosophila* olfactory sense organs, maxillary palpi, may respond to a specific unidentified odorant such as pheromone. In addition, positive selection on *Or33c* was detected between species. To further investigate the potential function of *Or33c* in *Drosophila melanogaster*, the expression level of *Or33c* between different populations and different sexes were compared, and sexual dimorphism of this gene had been observed. Sequence polymorphisms of *Or33c* and its tandemly duplicated genes, *Or33a* and *Or33b* (expressed on the other olfactory sense organs, antennae), of *D. melanogaster* populations were also compared to characterize how positive selection shapes the evolution of *Or33c* in *Drosophila*. Analyses of the DNA sequences showed that in both African and non-African populations there are significant signatures of positive selection in *Or33c*. *Or33c* had acquired *cis*-regulatory motifs required for the gene expression in the maxillary palp, the acquisition might involve the expression shift to different sensory organs and/or positive selection. Understanding the driving force of positive selection on *Or33c* will help us investigate the potential role of this gene in the detection of pheromonal CHs.

Keywords: cuticular hydrocarbon, *Drosophila melanogaster*, olfactory receptor, positive selection, sexual dimorphism

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Introduction

Dispersal history of *Drosophila melanogaster*

D. melanogaster has served as an important model system in molecular and evolutionary genetics. Biogeographical and systematic studies suggest a colonization history of *Drosophila melanogaster*. The cosmopolitan D. *melanogaster* originated from tropical Africa and colonized Europe and Asia approximately 10,000 to 15,000 years ago, and followed by a relatively recent migration to the Americans and Australia 150 years ago (David and Capy 1988; Lachaise et al. 1988). Through this process of population differentiation, many endemic variations have been generated, which could be detected by different molecular markers, including DNA sequence data, mtDNA, and microsatellites (Kreitman 1983; Kreitman and Aguadé 1986; Singh and Rhomberg 1987; Hale and Singh 1991; Agis and Schlötterer 2001; Baudry, Viginier, and Veuille 2004; Pool and Aquadro 2006; Nolte and Schlötterer 2008). Among these variations, some may contribute to nascent reproductive isolation, thus could shed light on our understanding towards speciation.

Reproductive isolation

Reproductive isolation could involve many complex mechanisms, including geography, ecology, behavior, and interactions between multilocus genotypes. As a

consequence, it could arise various forms and is conventionally categorized based on the stage in life cycle during which it has been observed: pre-mating, post-mating, pre-zygotic, and post-zygotic (Coyne and Orr 2004). It is worth noting that in many cases, especially allopatric ones, the reproductive isolation is just by-products of genetic divergence because there are no forces acting to enforce reproductive compatibility, instead of direct causes of speciation. Nonetheless, since the essential inquiry in evolution is "why and how organisms change", not "whether two groups can be regarded as two species", we could still extract hints from this kind of survey to answer some interesting questions like: Which factor plays a more important role in divergent process, stochastic drift or directional selection? And what kind of driving force may involve in selection, environmental adaptation or sexual interaction? The major aims of this study are trying to explore these questions in *Drosophila melanogaster* populations, in which some premating reproductive isolation could be observed.

Premating isolation

Premating isolation could be achieved through difference in habitats, temporal activity patterns (Marshall and Cooley 2000), modes of reproduction, and systems of mate recognition. In the cases of mate recognition, populations come to differ in signaling traits (of "chosen" individuals, which is usually male) and preferences (of

"choosy" individuals, which is usually female) for those traits. There are mainly two non-exclusive theories proposed to explain how this occurs: 1) In Fisher's runaway model, genes for female preference become genetically correlated with genes for male traits that are more attractive to females. Therefore, when genetic drift changes either the trait or the preference, the runaway sexual selection could cause behavioral isolation between different populations (Lande 1981). It is suggested this sort of mechanism may explain the spectacular divergence in plumage and display among groups of closely related species like the birds of paradise (Frith and Beehler 1998). 2) In sensory drive hypothesis, both male mating traits and perceptual systems underlying female preferences adapt to local environments (Endler 1992, 1993). Divergence occurs because local environments impose selection of various intensity and direction on mating signals aimed at potential mates and on sensory systems that acquire information on predators, prey and mates (Ryan 1998). Previous works on lizards and cichlid fishes have shown evidence supporting the hypothesis that adaptation to local light environments drives specializations of the visual and signaling systems, bring on habitat partitioning and speciation (Leal and Fleishman 2002; Seehausen et al. 2008).

In fruit fly *Drosophila*, the traits involved in mechanisms of isolation also comprise a variety of different types, such as pheromones, courtship song, shortened copulation time, genital morphology, and male/female sexual isolation (Coyne 1996a, b,

c; Coyne and Charlesworth 1997; Hoikkala et al. 2000; Zeng et al. 2000). The populations surveyed in this study contain African populations of *D. melanogaster* in and near Zimbabwe and non-African populations from other continents. The matings between Zimbabwe females and cosmopolitan males are typically very rare (Wu et al. 1995; Hollocher et al. 1997). Taking its asymmetry and the existence of hybrid zones in Africa into consideration, this incomplete premating isolation may indicate an incipient form of speciation (Wu et al. 1995; Ting, Takahashi, and Wu 2001). Analyzing the incipient speciation may benefit from being interfered by fewer secondary effects occurring after speciation, providing clues to clarify what kind of gene may involve in the primary phase of reproductive isolation.

Cuticular hydrocarbons and peripheral olfactory system in *Drosophila*

In *D. melanogaster*, males can distinguish the gender of other flies even when most auditory and visual signals are removed. However, this ability is lost when encountered with *desat1* mutant line, whose males and females have very similar cuticular hydrocarbon (CH) profiles (Marcillac, Grosjean, and Ferveur 2005). It is revealed that sexual dimorphic CHs serve as pheromones for mate discrimination, and may involve in sexual isolation just as in *D. simulans*/*D. sechellia* case (Coyne, Crittenden, and Mah 1994). Previous studies showed that *D. melanogaster* populations

from Africa and other continents are different in cuticular hydrocarbons; and a mutation in the promoter of *desat2*, a duplicate of *desat1* coding for another desaturase, is correlated with the assortative mating pattern described above (Takahashi et al. 2001). In a pheromone communication system, tuning of receiver could be correlated with changes in sender's signal, as suggested in *Ostrinia* moth (Baker 2002). Thus, it would be intriguing to survey whether there are some corresponding modifications in receiver terminal, the olfactory receptors.

Many insects have two bilaterally symmetrical pairs of olfactory organs, the antennae and the maxillary palps. The surfaces of these olfactory appendages are covered with sensory hairs, called sensilla, containing olfactory receptor neurons (ORNs) dendrites that are specialized to detect odors. In *D. melanogaster* adults, each antenna contains about 1200 ORNs, and each maxillary palp contains about 120 ORNs (Stocker 1994; Shanbhag, Müller, and Steinbrecht 1999; Shanbhag, Müller, and Steinbrecht 2000). Each sensillum contains dendrites of one to four ORNs. The olfactory sensilla of the antenna can be subdivided into three major morphological and functional classes: club-shaped basiconic sensilla, long and pointed trichoid sensilla, and short, peg-shaped coeloconic sensilla, whereas the olfactory sensilla of the maxillary palp consist entirely of basiconic sensilla.

Search of *D. melanogaster* genome using bioinformatics methods predicted that there are 62 OR proteins encoded by families of 60 Or genes through alternative splicing (Robertson, Warr, and Carlson 2003). *Drosophila* OR proteins are highly diverse and have no similarity to mammalian odorant receptors. However, closely linked genes often share a higher degree of sequence similarity, suggesting that some *Or* genes are likely to have undergone recent duplication events to generate clusters of *Or* genes.

There is a segregation of gene expression pattern between the two major olfactory organs: ORs expressed in the antenna are not expressed in the maxillary palp and vice versa. In situ hybridization showed that there are 32 Or genes expressed on the antenna and seven Or genes expressed on the maxillary palp (Vosshall, Wong, and Axel 2000).

Pheromonal communication and *Or* genes

In the past several years, the pheromone detection and processing in *Drosophila* male mating behaviors have been the focus. The best understood male pheromone is *cis*-vaccenyl acetate (cVA), a volatile compound modulating both male and female behaviors (Bartelt, Schaner, and Jackson 1985; Xu et al. 2005; Ejima et al. 2007; Kurtovic, Widmer, and Dickson 2007). One olfactory receptor expressed on antenna, *Or67d*, is required for cVA detection (Kurtovic, Widmer, and Dickson 2007). LUSH, an odorant binding protein, will bind cVA to activate *Or67d* (Xu et al. 2005). Then the

activated *Or67d* ORNs will target to a glomerulus called DA1 in the antennal lobe and pass the cVA signal to the corresponding DA1 projection neurons (PNs) (Couto, Alenius, and Dickson 2005; Schlief and Wilson 2007; Datta et al. 2008). Although both Or67d ORNs and DA1 PNs respond to cVA equally in males and females, the DA1 PNs form sex-specific arborization in the protocerebrum, suggesting that the cVA signal may process differently in males and females (Datta et al. 2008). In addition to *Or67d*, *Gustatory receptor 32a* (*Gr32a*) and *Gr68a* on the legs, *Or47b*, *Or65*a and *Or88a* on the antenna have also been proved d to have function of pheromone detection.

Of 60 Or genes, *Odorant receptor 33c* (*Or33c*), *Or42a*, and *Or85e* were suggested under positive selection across eight ecologically diverse species of *Drosophila* including *D. melanogaster*, *D. mauritiana*, *D. simulans*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. ananassae*, and *D. pseudoobscura* by using maximum likelihood estimates of dN/dS ratios (Tunstall et al. 2007). Among these three genes, *Or33c* and *Or85e* are coexpressed in the same ORN class on the maxillary palp. The electrophysiological experiments showed that responses of *Or33c* receptor to 150 odorants are much weaker than *Or85e* receptor, suggesting that *Or33c* may respond to a specific unidentified odorant such as pheromone (Goldman et al. 2005). To further characterize how positive selection shapes the evolution of *Or33c* in *Drosophila* and whether the positive selection of *Or33c* is related to the potential role on the detection of CH pheromones,

sequence polymorphisms of *Or33c* and its tandemly duplicated loci, *Or33a* and *Or33b*, of *D. melanogaster* populations were compared.

Materials and Methods

Fly Stocks

Twenty-six African strains and 14 non-African strains of *Drosophila melanogaster* were used in the sequence analyses. African strains were collected from Zimbabwe (ZS2, ZS6, ZS8, ZS11, ZS29, ZS30, ZS49, ZS53, and ZS56 in Shegwa Wildlife Reserve; ZH12, ZH18, ZH21, ZH23, ZH32, and ZH34 in Harare), Northern Zambia (LA2, LA20, and LA66), Botswana (OK17 and OK91), Ivory Coast (Tai), and Malawi (MW6, MW11, MW28, MW56, and MW63). The cosmopolitan lines were from several different continents, including Europe (FR in France), North America (Canton-S in Ohio; HG in California; Id in Indiana; Oahu in Hawaii; OR in Oregon; mm1 in North Carolina; M97 in Miami), South America (Ica in Peru), Asia (NFS6 in Israel; QD12 and QD18 in Japan; TWN in Taiwan), and Australia (Yv). ZS6, ZS8, ZS11, ZS30, ZH18, ZH23, ZH32, LA20, LA66, FR, CS, HG, Id, and Ica lines were isogenic for the second and third chromosomes. For the rest of lines which are isofemale lines, one male from each lines was crossed with females carrying the deficiency *Df(2L)Prl, Prl*^{1} *nub*^{Prl}*/CyO*, which covers cytological positions 32F1-3 to 33F1-2; hemizygotes of *Or33* genes could be selected from the progeny. All flies were kept in vials containing the standard yeast-cornmeal-agar medium and maintained at 25°C with humidity control under a

12h-12h light-dark cycle.

Total RNA extraction

Adult flies (three to five days old) were put into an empty vial for at least two hours to void yeast before head dissection. Adults were dissected in phosphate buffered saline to separate heads from bodies. Total RNA was extracted from heads with Trizol® reagent according to the manufacturer's instructions (Invitrogen). RNA pellets were resuspended in diethyl pyrocarbonate (DEPC)-treated water. To prevent genomic DNA contamination, the samples were treated with Deoxyribonuclease I (Invitrogen) and stored at -80°C.

Quantitative RT-PCR

A 1-μg aliquot was used as template for cDNA synthesis, employing the SuperScript™ III First-Strand Synthesis system (Invitrogen) and oligo(dT) primers. The cDNA was stored at -80°C. Quantitative RT-PCR assays were performed by the Universal Probe Library (UPL) system (Mouritzen et al. 2005). Primers and probes for determination of *Or33c* and endogenous control *rp49* expression levels were designed using the Roche Applied Science Universal Probe Library with online tools (http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) (table 1). Each 25 μl reaction contained 12.5 μl of 2X FastStart Universal Probe Master (ROX), 100 nM

Universal Probe, 200 nM of each primer, and 50 ng cDNA. Reactions were subjected to the following cycles on the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems): step1- 95°C for 10 minutes; step 2- 95°C for 10 sec, 60°C for 1 minutes, repeat step 2 for 39 additional cycles. Data analysis was based on the relative expression level of *Or33c* to the reference gene, *rp49*, with a normalized calibrator method described in manufacture's instructions (Roche Applied Science). All reactions were performed in triplicate, and repeated four times.

Table 1

Primers and probes designed for quantitative RT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')	Probe
Or33c	CATCAGCGTTTGCTGGATTA	TCACCAGGTTGTGGAATCG	# 132
rp49	CTTCATCCGCCACCAGTC	CGACGCACTCTGTTGTCG	# 117

DNA extraction, PCR amplification, and sequencing

DNA was extracted from single fly following modified Puregene DNA Purification Kit protocol (Gentra). Gene-specific primers (table 2 and appendix) were used to perform PCR amplification. DNA sequences of PCR-amplified fragments were sequenced on ABI PRISM® 96-capillary 3700 xl DNA Analyzer.

Table 2

Primers designed for PCR amplification

Forward Primer	Sequence (5'-3')	Reverse primer	Sequence (5'-3')	Fragment size (bp)
*Or33b*_ -1193F	CAAGCAACCGCCGCAATCTA	*Or33b*_-94R	CCACATAGTTACGCCCATCA	1119
Or33b -563F	AAAGCAGGTGGTATTGTTGGCATCG	*Or33b*_-32R	ACAATTGAAAAGGTCCTGCGGCATT	556
Or33b -877F	TAATACGCCTACTTCGCAGCACTT	*Or33b*_589R	ACCAGCAACCACGCAAAATGTCAT	1489
*Or33b*_437F	TTTTCGGCGGTGGACATAAGC	*Or33b*_1284R	CCAAAGTGAAGAAGGAGTAGG	868
*Or33c*_-1021F	TAAATGAAGGTGTTGTAGGA	*Or33c*_15R	TGGACGATAAAAACTAAGACTG	1057
*Or33c*_-76F	ATTTCTCGCAGTGTTTCCTCTC	*Or33c*_1365R	AACTGTTTATTTGCGTATC	1459

Linkage disequilibrium

To determine whether the association between polymorphic sites are significant or not, the linkage disequilibrium (LD) graphs measured by r^2, which is equal to the square of the correlation coefficient between the alleles at two loci (Hill and Robertson 1968), were constructed using data calculated form DnaSP version 5 (Librado and Rozas 2009).

Sequence analyses

Multiple sequence alignments were performed by SeqMan, EditSeq, and MegAlign software with ClustalW method (Lasergene, DNAStar, Madison, WI). Several neutrality tests were carried out by DnaSP version 5 (Librado and Rozas 2009). Sites containing alignment gaps were not used in all the tests.

In the analysis of divergence between species, only coding regions were used for the McDonald and Kreitman test (McDonald and Kreitman 1991). This test is based on a comparison of synonymous and non-synonymous variation within and between species. *D. simulans* was used as outgroup (sequence is available from Flybase). Under the neutral theory, the ratio of replacement to synonymous fixed substitutions (R/S) should be the same as the ratio of R/S within species.

Transmembrane (TM) helix prediction was carried out with the program HMMTOP 2.0 (Tusnády and Simon 2001) and visualized by TMRPres2D (Transmembrane Protein Re-presentation in 2 Dimensions tool), which automates the creation of graphical images and models of TM proteins (Spyropoulos et al. 2004).

The following three tests are derived from θ estimated from different parts of frequency spectrum which response to the selection differently. Tajima's *D* test (Tajima 1989) is based on the normalized difference between the number of segregating sites (θ) and the average number of nucleotide differences (π). At equilibrium between genetic drift and selection, the expected value of *D* is close to zero. *D* is negative when there is a relative excess of rare alleles in a folded frequency spectrum.

Fu and Li's *D** and *F** test (Fu and Li 1993) are according to the expected total length of the external and internal branches. Fu and Li's *D** test is based on the differences between the number of singletons and the total number of mutations. Fu and Li's *F** test is based on the differences between the number of singletons and k, the average number of nucleotide differences between pairs of sequences. D* and *F** are negative when there is a relative excess of mid-frequency alleles in a folded frequency spectrum.

Fay and Wu's *H* test (Fay and Wu 2000) is used to measure an excess of high

compared intermediate frequency variants. This test is based on the differences between k and θ_H, an estimator based on the frequency of the derived variants. Negative H value means an excess of high-frequency-derived alleles relative to the standard neutral model.

Results

Expression level of *Or33c*

To examine whether the expression levels of *Or33c* are different between the African and non-African populations, four lines from the non-African (FR and Canton-S) and the African (Z30 and Z56) populations were divided into two groups (FR vs. Z30; Canton-S vs. Z56) to compared by quantitative RT-PCR. The result showed that the expression levels between these two populations have no significant difference (two-tailed Student's t-test, $P > 0.1$). Interestingly, the expression levels in males were significantly higher than in females (fig. 1).

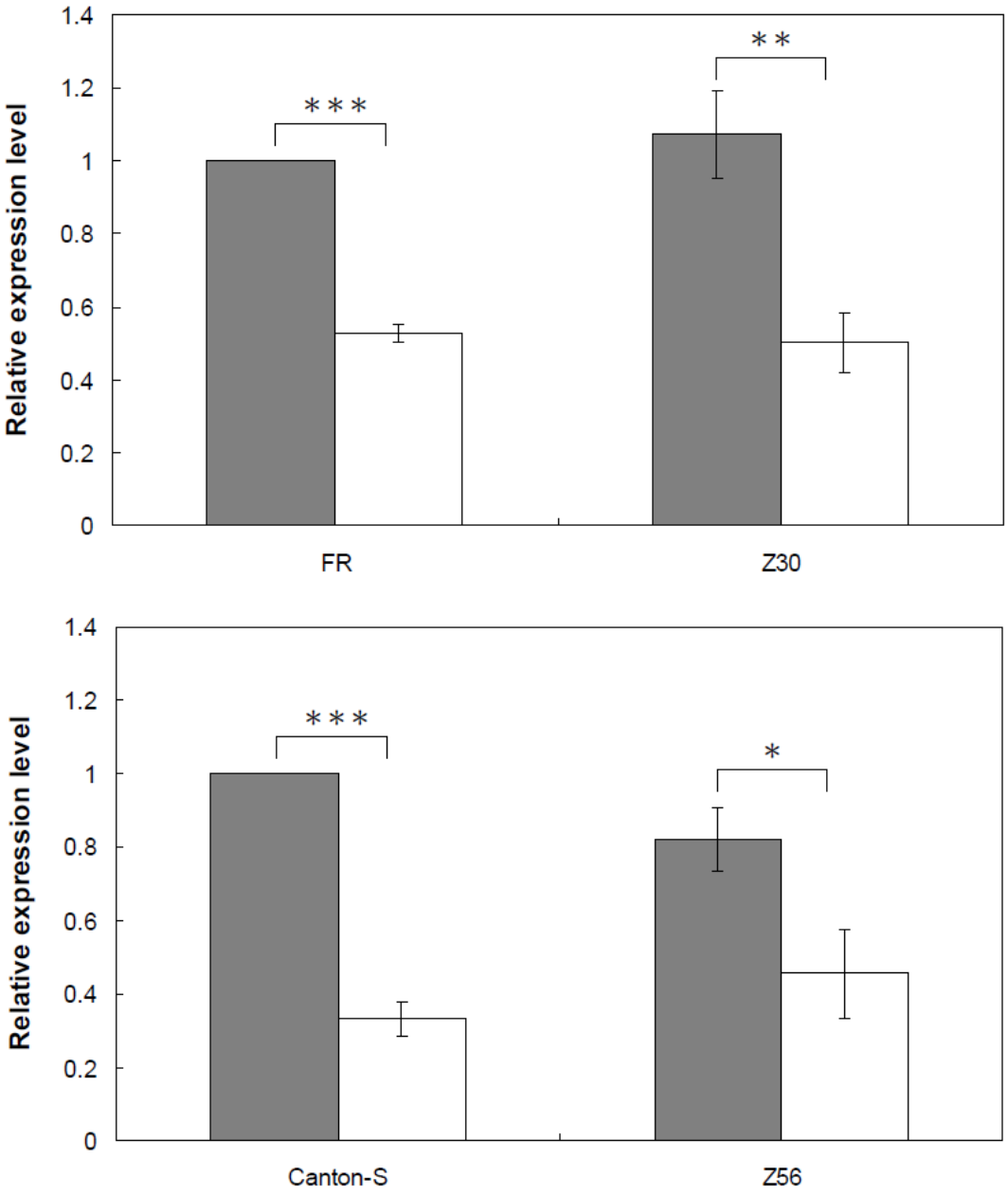


Fig. 1.—Expression levels of *Or33c* in *D. melanogaster* populations, as quantified by real-time RT-PCR. All values are normalized against an internal control (*rp49*). Solid bars, males; open bars, females. *$P < 0.05$, **$P < 0.01$, and *** $P < 0.001$, two-tailed Student's t-test.

Nucleotide polymorphism in African and non-African populations

The sequenced region spanning *Or33a*, *Or33b*, and *Or33c* genes consists of 4185 bp in length. Fig. 2 summarizes the distribution of nucleotide sequence polymorphism compared with D. *simulans* as outgroup, including a total of 399 polymorphic sites in 14 non-African strains and 26 African strains. Summary statistics of molecular variation are shown in table 3. At different regions, k ranges from 5.874 to 17.145, θ_w and π range from 0.01 to 0.024 in the African populations; k ranges from 4.462 to 15.725, θ_w and π range from 0.007 to 0.014 in the non-African populations. Nucleotide diversity of *Or33* genes was shown in fig. 3. The result showed that the nucleotide polymorphism in the African population is higher than in the non-African population.

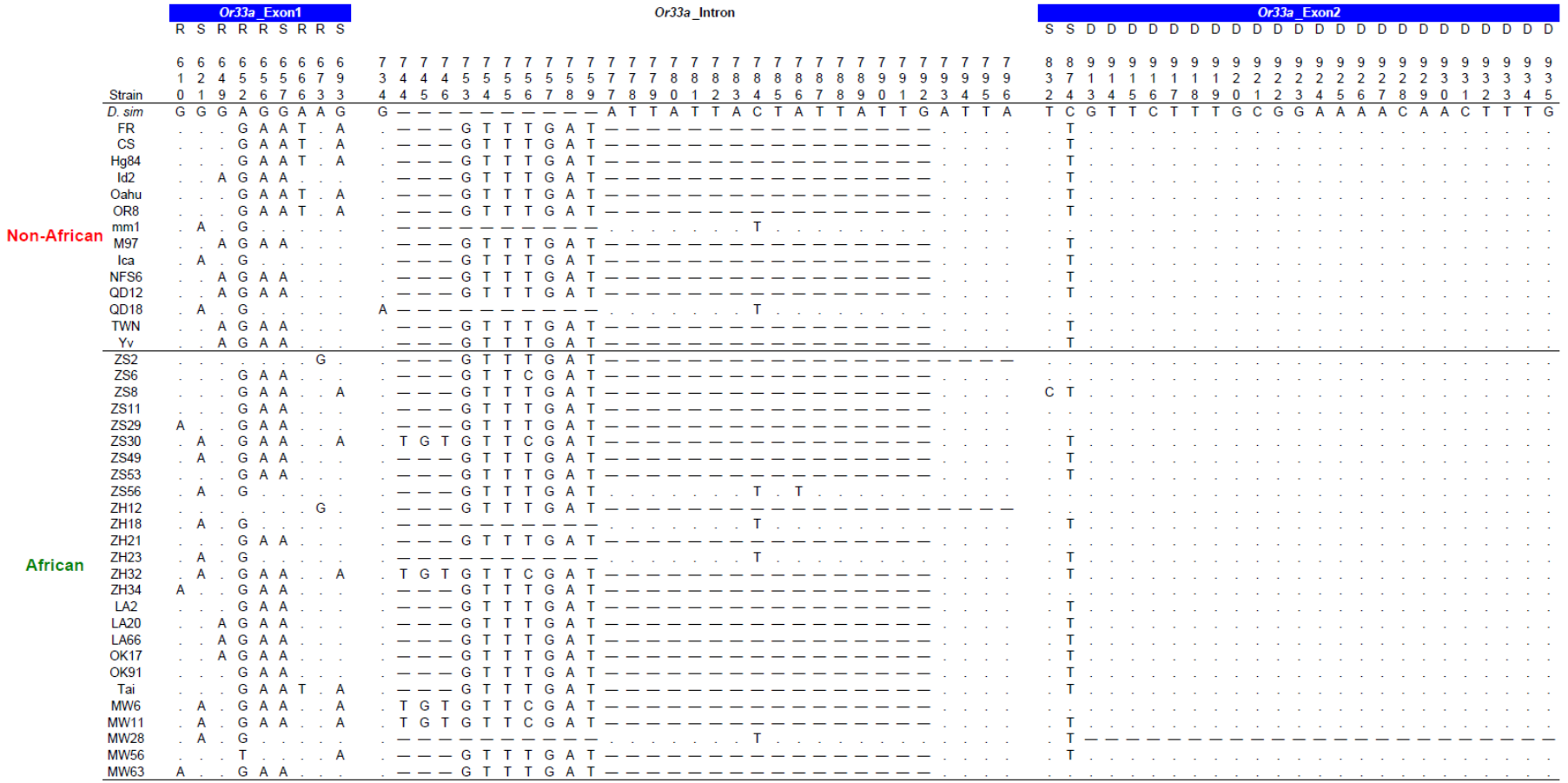


Fig. 2.—Polymorphic sites of *Or33* genes in the populations of *D. melanogaster*. Polymorphisms are numbered from the initiation codon of each gene. Dots represent the same nucleotide as in the *D. simulans* sequence. Dashes represent alignment gaps in nucleotide sequence. R and S stand for replacements and synonymous, respectively.

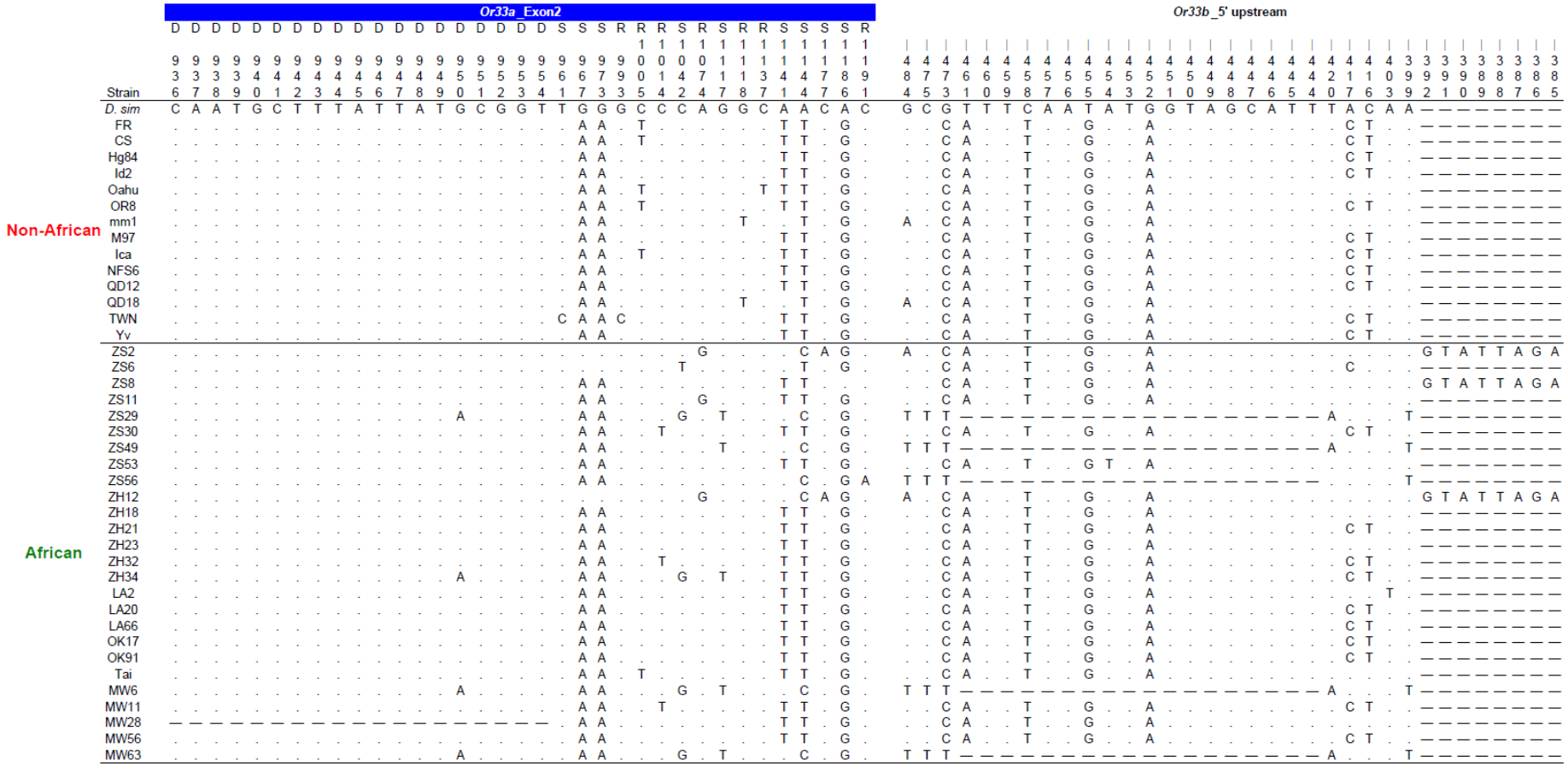
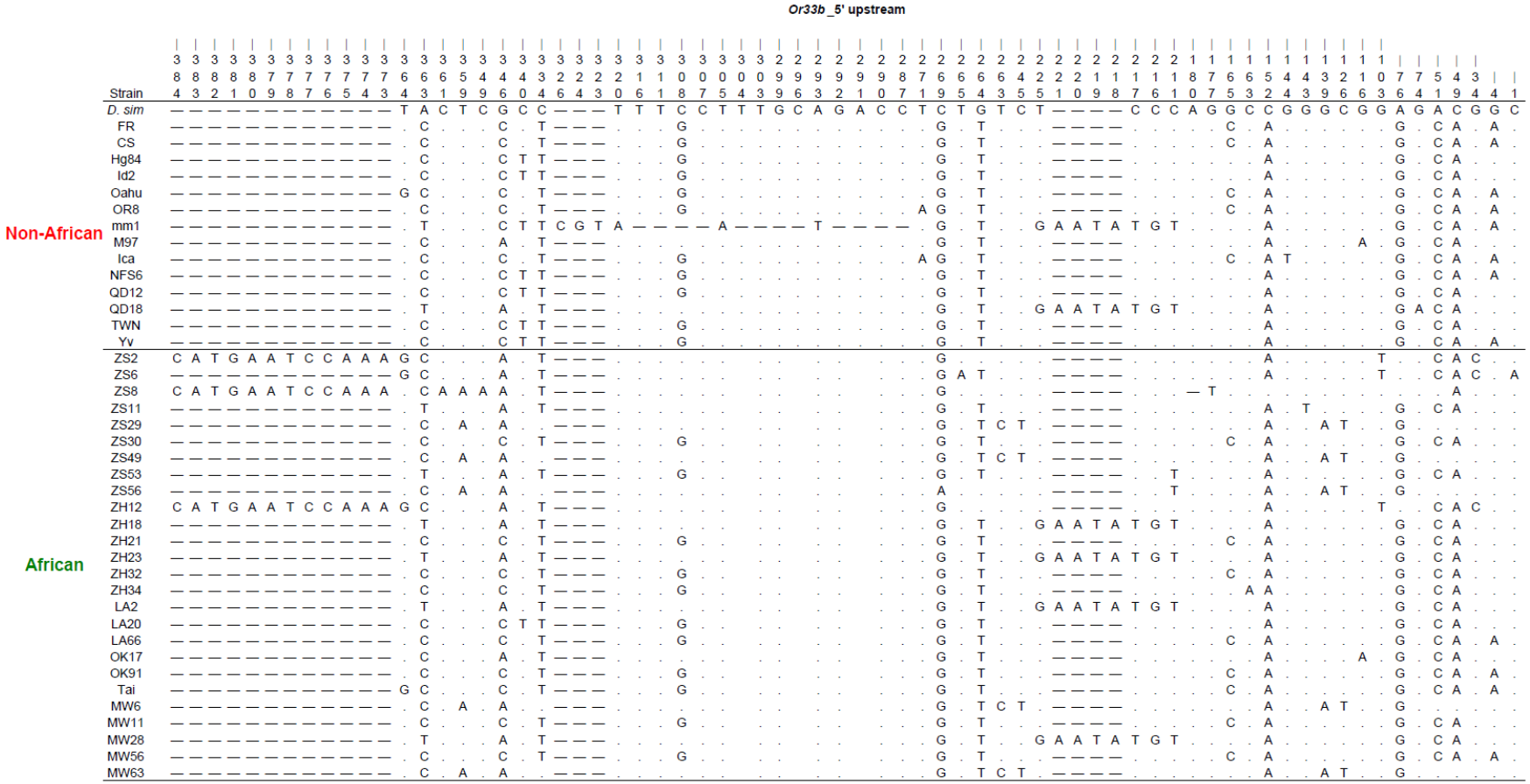


Fig. 2.—(Continued)



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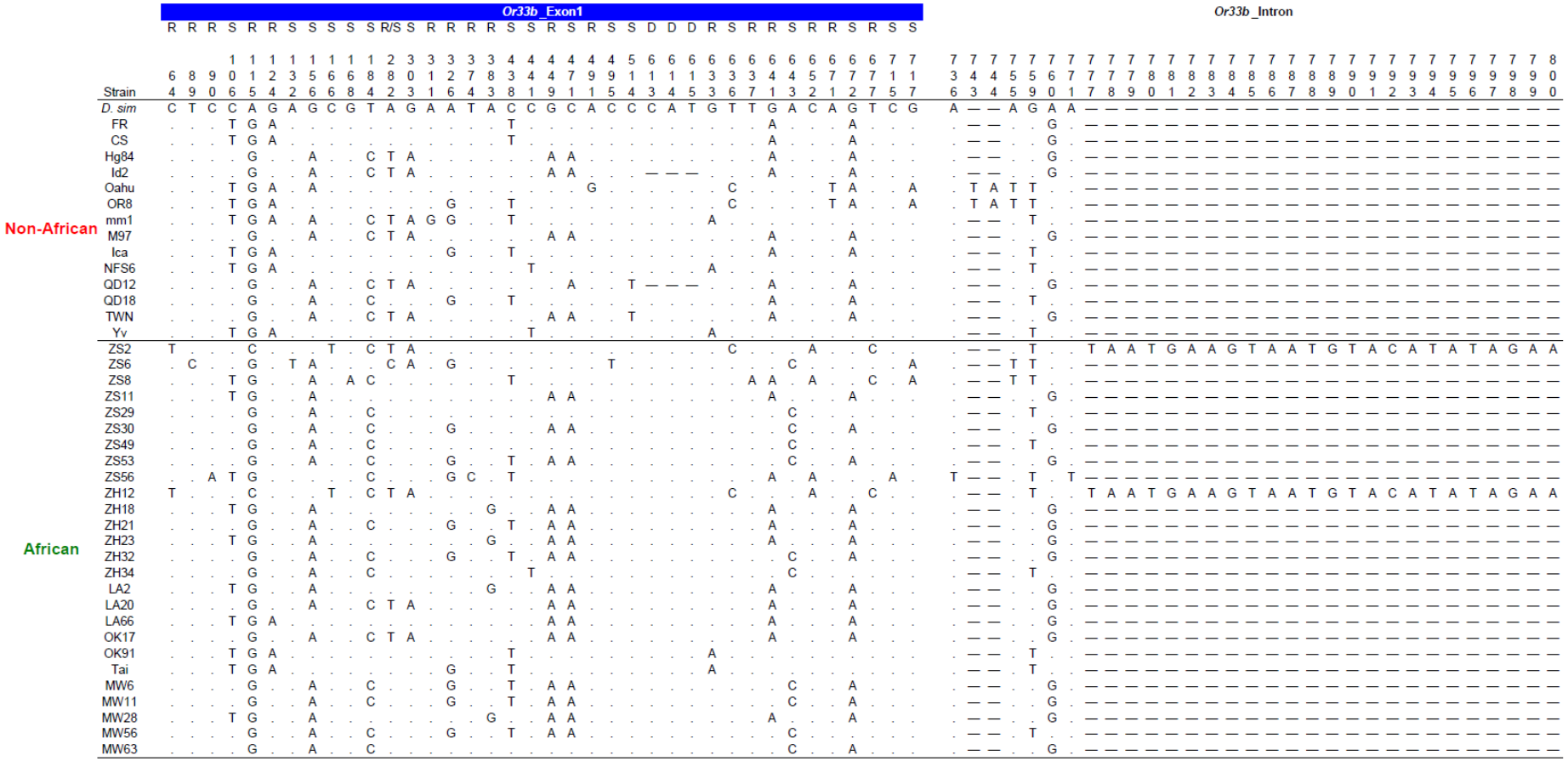
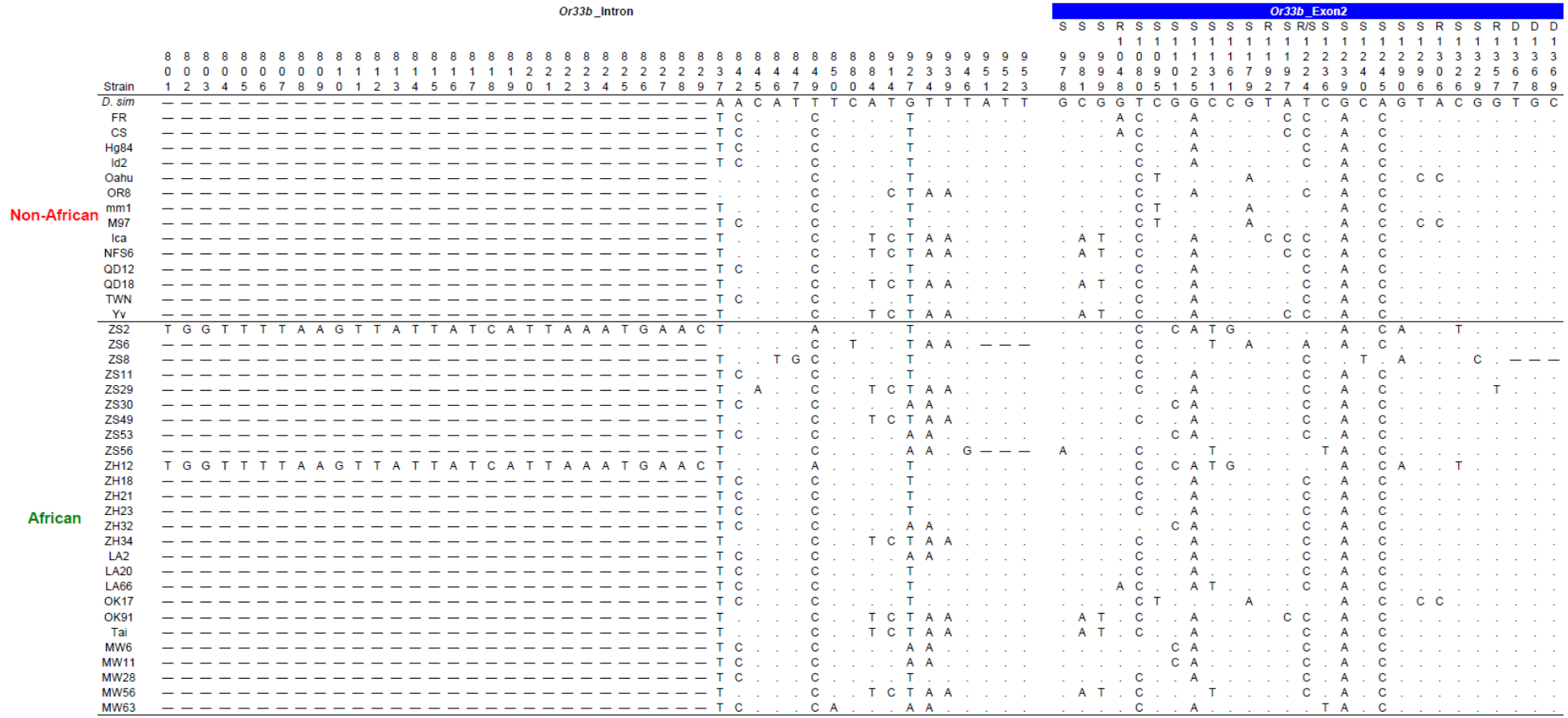


Fig. 2.—(Continued)



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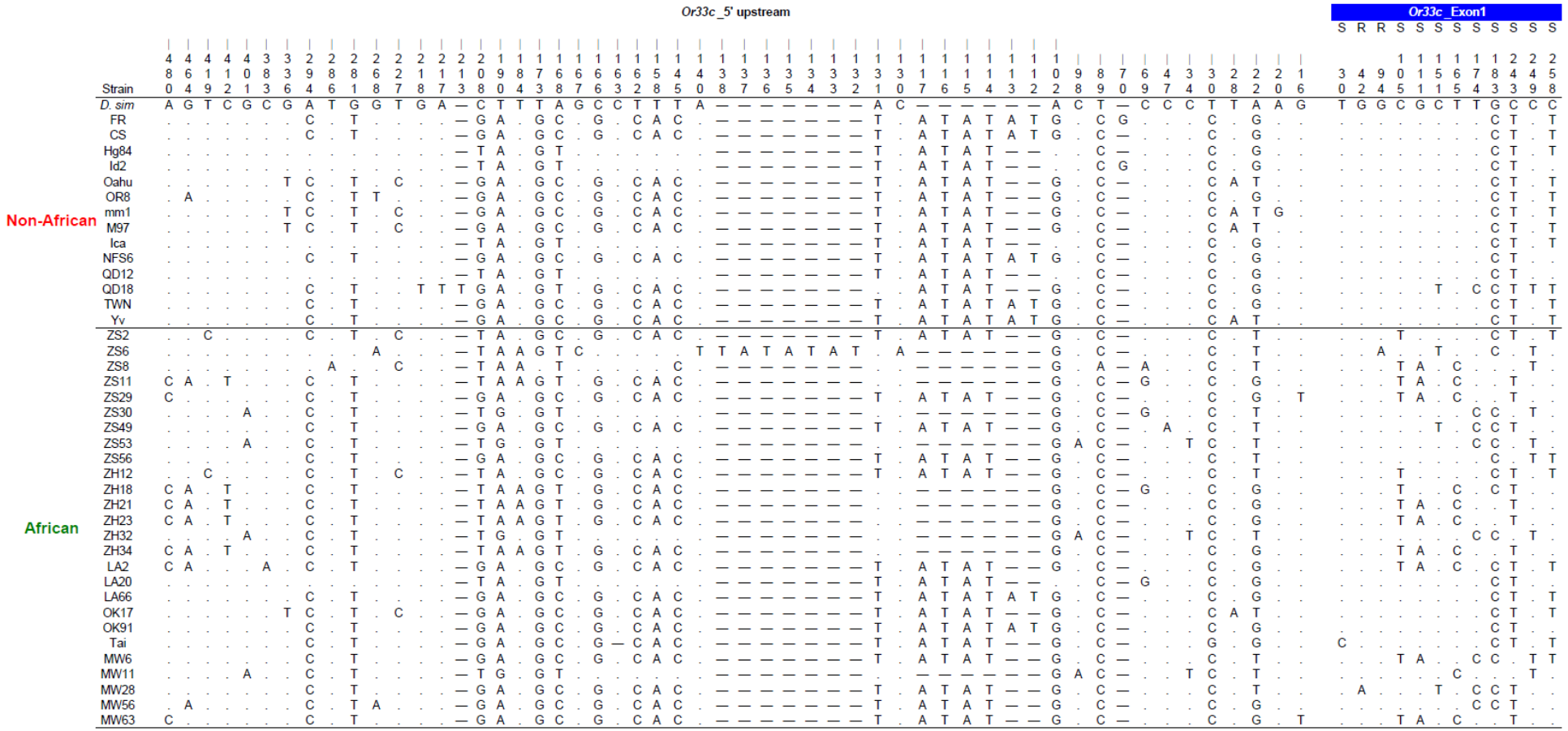


Fig. 2.—(Continued)

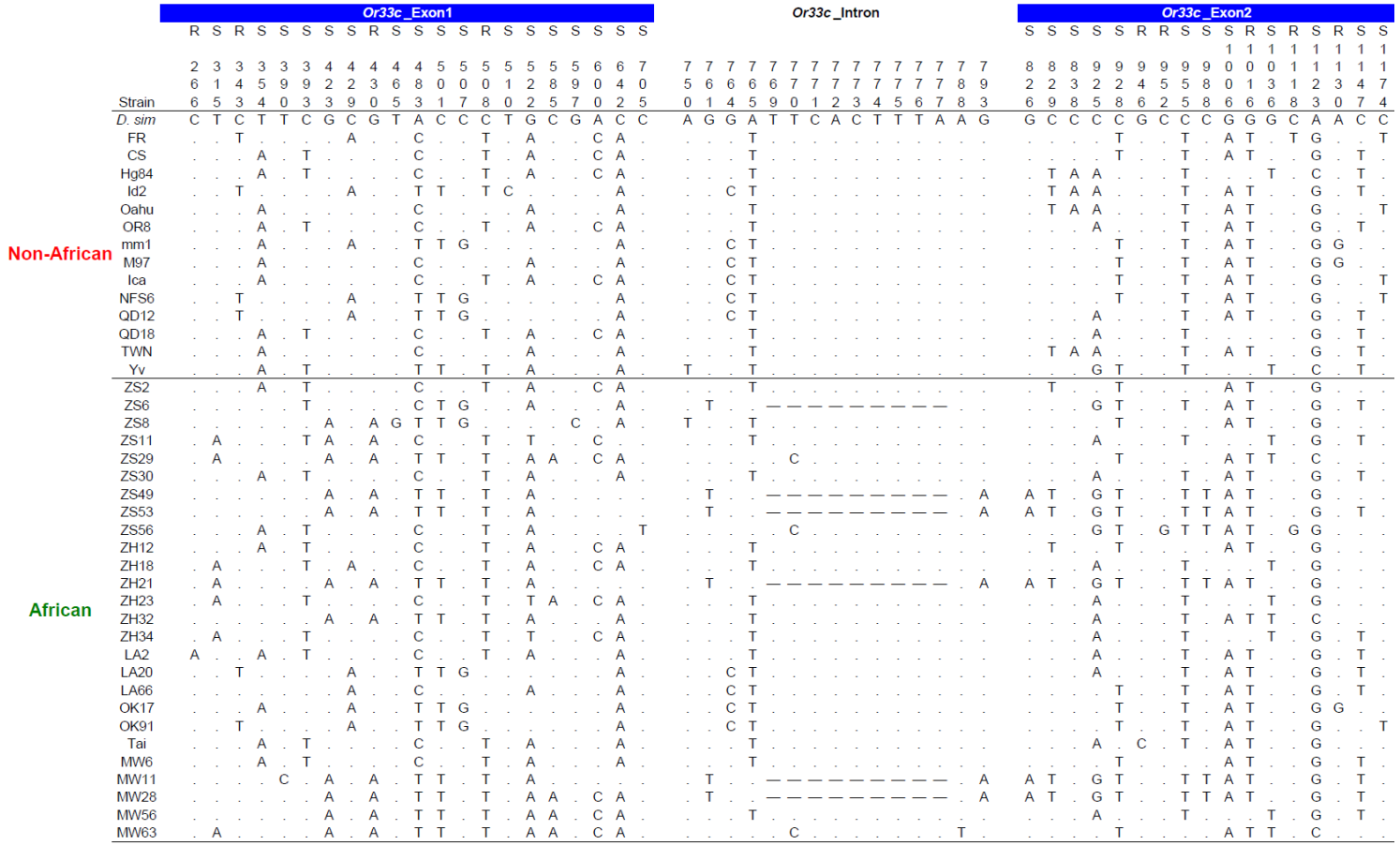


Fig. 2.—(Continued)

Table 3

Summary statistics of molecular variation for *Or33* genes

Gene	Length	*S*	k	θ_w	π
African (N = 26)					
Or33a	661	23	5.874	0.010	0.010
Intergenic region	494	41	10.677	0.024	0.024
Or33b	1371	73	16.551	0.013	0.015
Intergenic region	482	36	8.877	0.019	0.020
Or33c	1177	54	17.145	0.015	0.012
Non-African (N = 14)					
Or33a	661	14	4.462	0.007	0.007
Intergenic region	494	20	5.396	0.012	0.014
Or33b	1371	40	15.725	0.012	0.010
Intergenic region	482	19	6.473	0.014	0.013
Or33c	1177	29	10.967	0.009	0.008
African and non-African (N = 40)					
Or33a	661	28	5.576	0.009	0.011
Intergenic region	494	46	9.031	0.021	0.025
Or33b	1371	78	16.642	0.013	0.014
Intergenic region	482	39	8.259	0.018	0.020
Or33c	1177	56	15.844	0.014	0.011

Note.—*S*, number of segregating sites; k, the average number of nucleotide differences; θ_w, Watterson's (1975) estimates of the mutation parameter; π, nucleotide diversity.

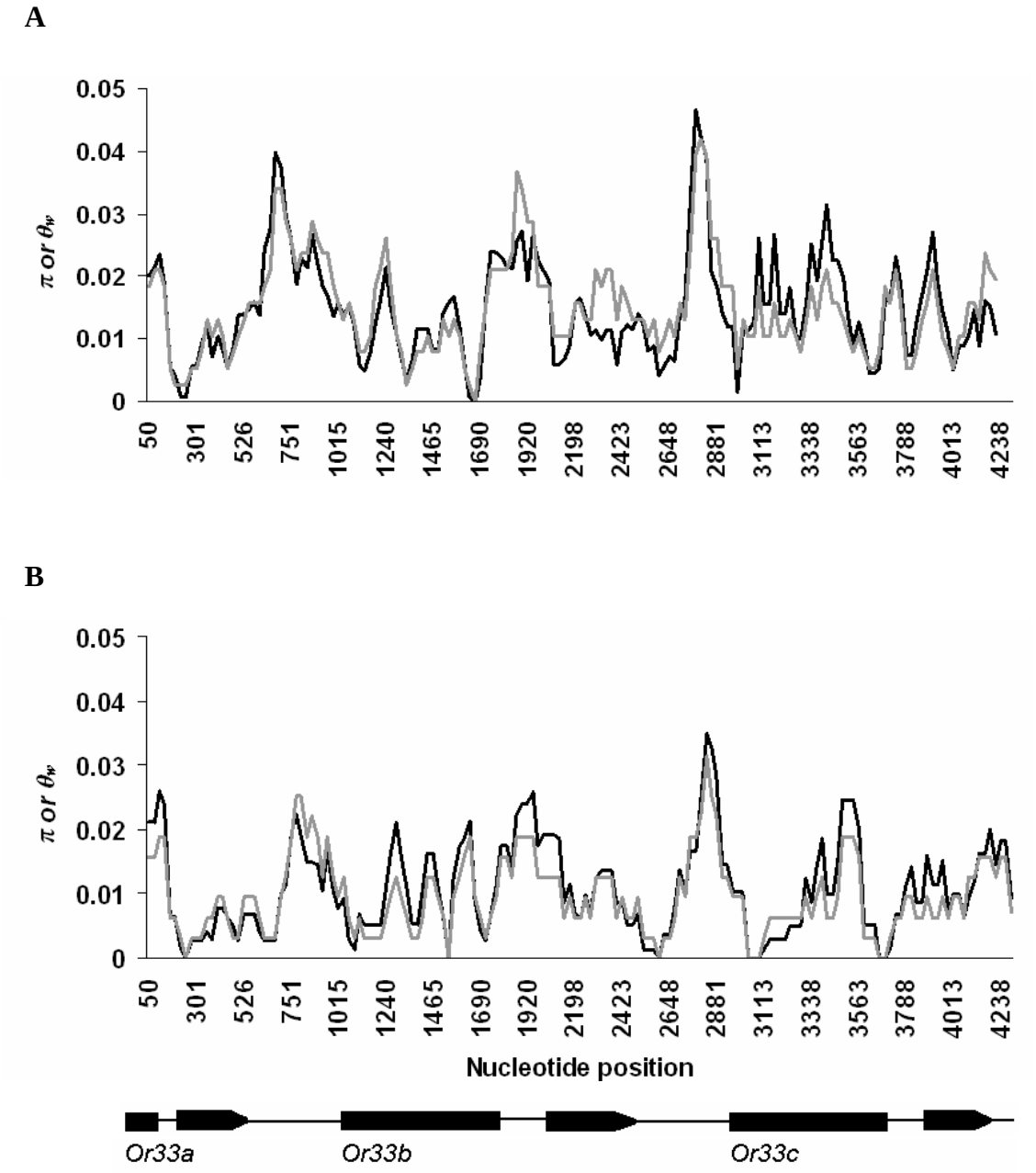


Fig. 3.—Sliding window plots of π (black lines), and θ_w (gray lines) along *Or33* genes sequence in African (A) and non-African (B) populations of *D. melanogaster*. A window length of 100 bp moved in the steps of 25 bp along the sequence was used. Gene structure of *Or33* genes was indicated by black boxes (exons) and thin lines (introns) at the bottom of the graph.

Linkage disequilibrium

The derived non-African population showed a higher level of linkage disequilibrium than the ancestral African population (fig. 4).

McDonald and Kreitman test

The DNA sequence variations of *Or33a*, *Or33b*, and *Or33c* coding regions were analyzed separately by the McDonald and Kreitman test (table 4). The evolutionary patterns of *Or33a*, *Or33b*, and *Or33c* revealed by the replacement to silent sites ratio (R/S) are different. In *Or33a* and *Or33b*, the R/S ratios between species (*D.melanogaster* and *D. simulans*) and within species (*D. melanogaster*) are not significantly different. Whereas in *Or33c*, the R/S between species is significantly higher than that within species. These patterns are similar in both the African population and the non-African population, suggesting that positive selection operating on *Or33c* when *D. melanogaster* diverged from *D. simulans*.

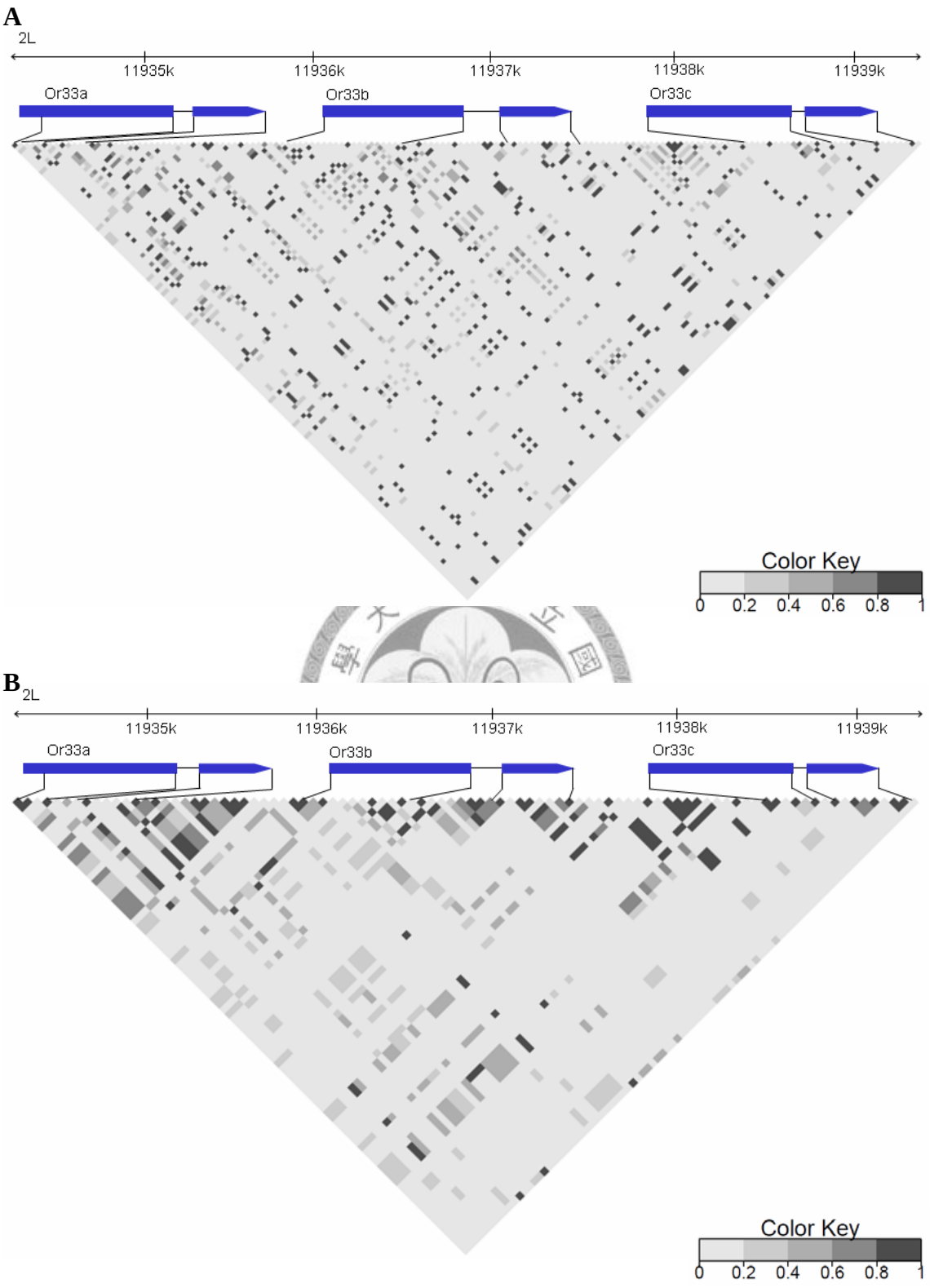


Fig. 4.—Linkage disequilibria of *Or33* genes in the African (A) and the non-African (B) populations of *D. melanogaster*. The degree of LD estimated by r^2 is shown by different shades.

Table 4

McDonald and Kreitman test for *Or33* genes

	Fixed differences (between species)		Polymorphic differences (within species)		
	R	S	R	S	*G* Value (*P* value)
African (N = 26)					
Or33a	7	9	11	14	0.000 (0.987)
Or33b	15	26	19	37	0.073 (0.787)
Or33c	20	22	11	39	**6.755 (0.009)**
Non-African (N = 14)					
Or33a	8	12	7	6	0.609 (0.435)
Or33b	15	29	12	19	0.168 (0.682)
Or33c	20	26	5	23	**5.398 (0.020)**
African and non-African (N = 40)					
Or33a	7	9	14	15	0.085 (0.771)
Or33b	15	26	23	38	0.013 (0.909)
Or33c	20	22	12	41	**6.565 (0.010)**

Note.—R and S stand for the number of amino acid replacements and silent changes, respectively. Significant *P* values (less than 5%) are in bold.

Predicted transmembrane helices

Among the fixed nucleotide changes between *D. melanogaster* and *D. simulans*, there are four sites involve in polarity changes (fig. 5). All of these four sites are located in the loop regions of the Or33c protein. The sites on the extracellular surface may involve in ligand binding, whereas intracellular sites may involve in signal transduction. The HMMTOP 2.0 prediction result showed that Or33c protein has the regular G protein-coupled receptor (GPCR) topology with the extracellular N-terminal. This topology is different to the novel *Drosophila* chemoreceptor topology proposed by Benton et al. (2006), which the N-terminal is in the intracellular surface. According to the regular GPCR topology, the four sites changing amino acid polarity are on the intracellular surface of the protein.

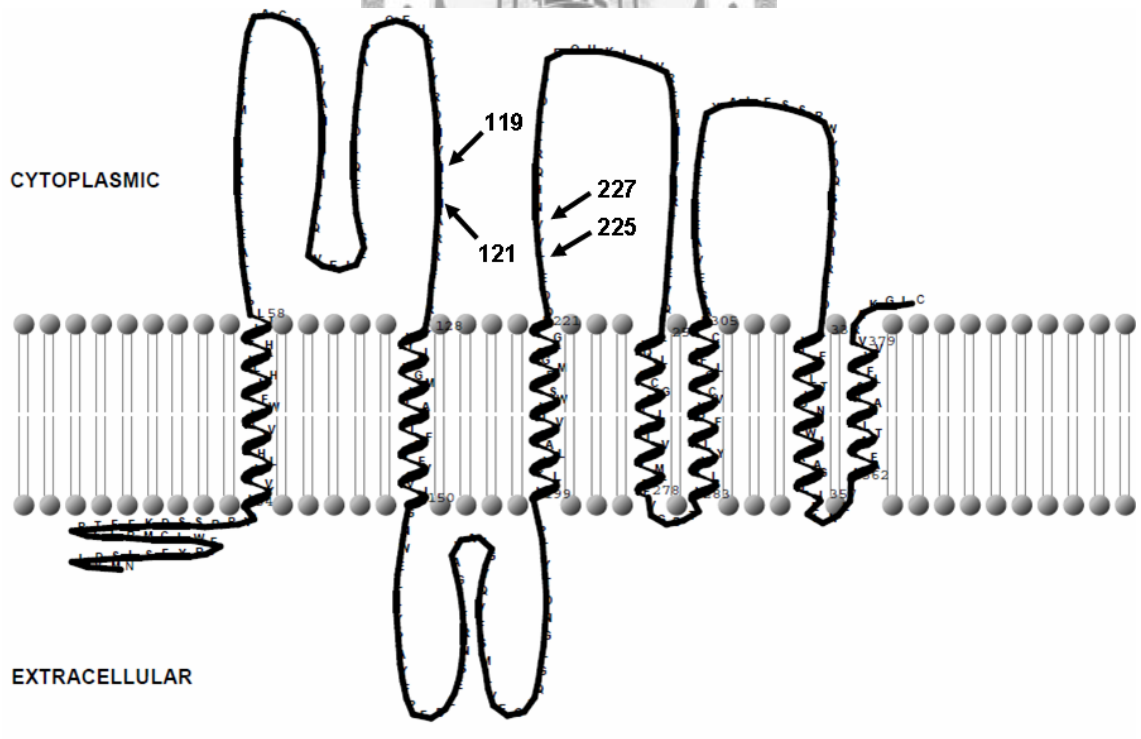
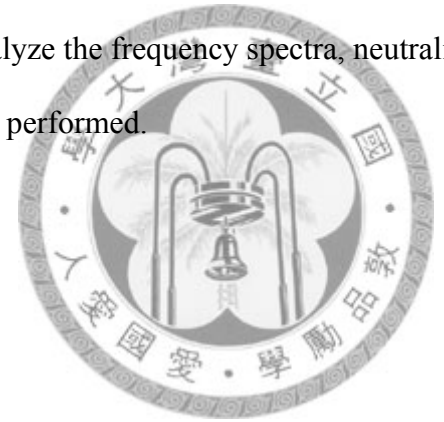


Fig. 5.—Predicted topology of Or33c protein. The arrows indicate the sites involving polarity changes.

Frequency spectrum

The goodness-of-fit test revealed that the frequency spectra on the non-coding region of *Or33b*, and *Or33c* in the African population significantly departures from neutrality (P = 0.013 and 0.033, respectively). In the non-African population and the combination of African and non-African populations, *Or33c* significantly deviate from the neutrality (P = 0 and 0.01, respectively). The synonymous sites frequency spectra of *Or33a* and *Or33c* are significantly higher than the neutral expectation in the African population (P = 0 and 0.009, respectively) and the combination of African and non-African populations (both P = 0). In the non-African population, *Or33a* and *Or33b* significantly departures from neutrality (P = 0.003 and 0.017, respectively) (figs. 6-8). To further analyze the frequency spectra, neutrality tests based on frequency spectrum were performed.



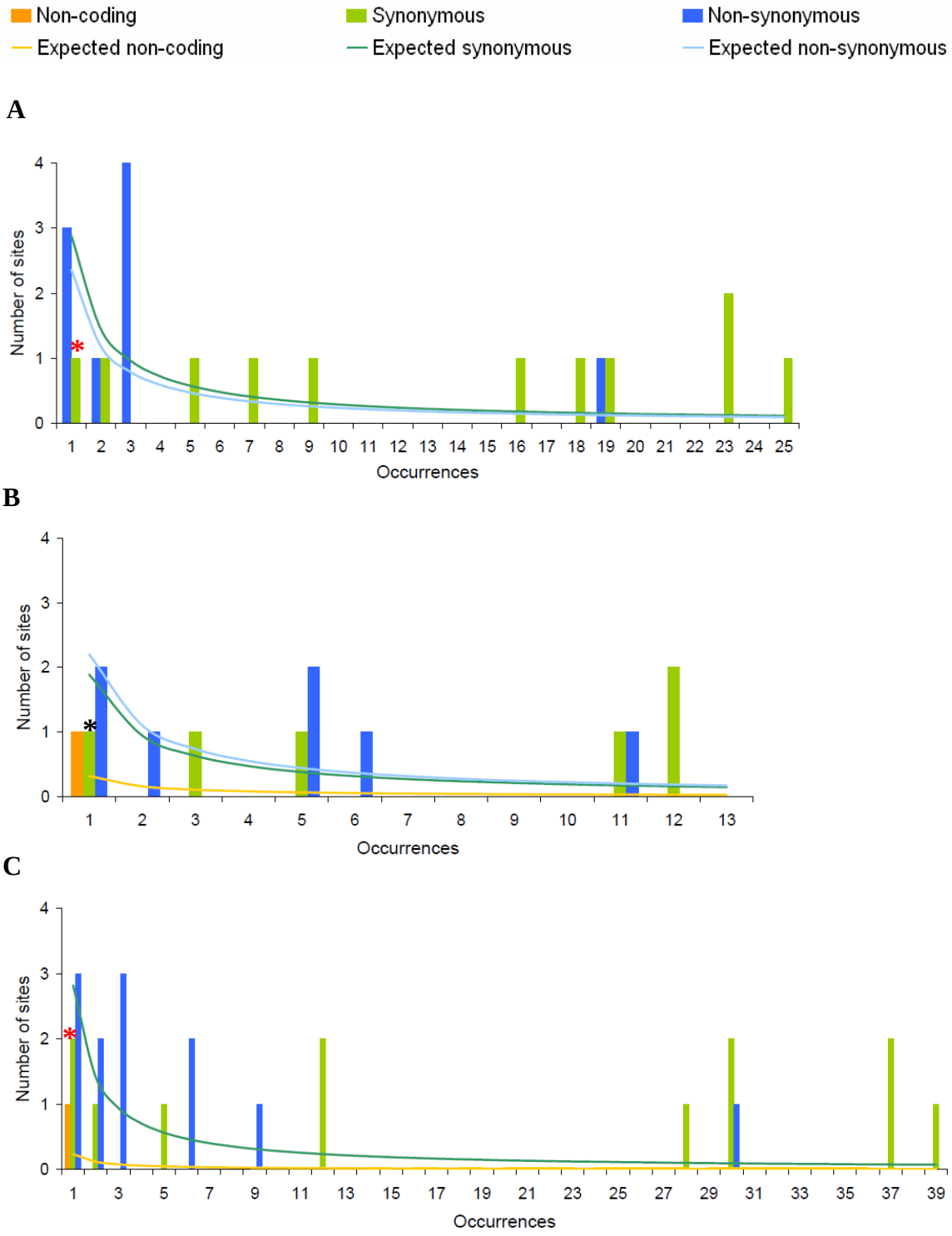


Fig. 6.—Frequency spectra of *Or33a* gene in African (A), non-African (B) populations, and the combination of African and non-African populations (C) of *D. melanogaster*. Black star, $0.001 < P < 0.01$; red star, $P < 0.001$.

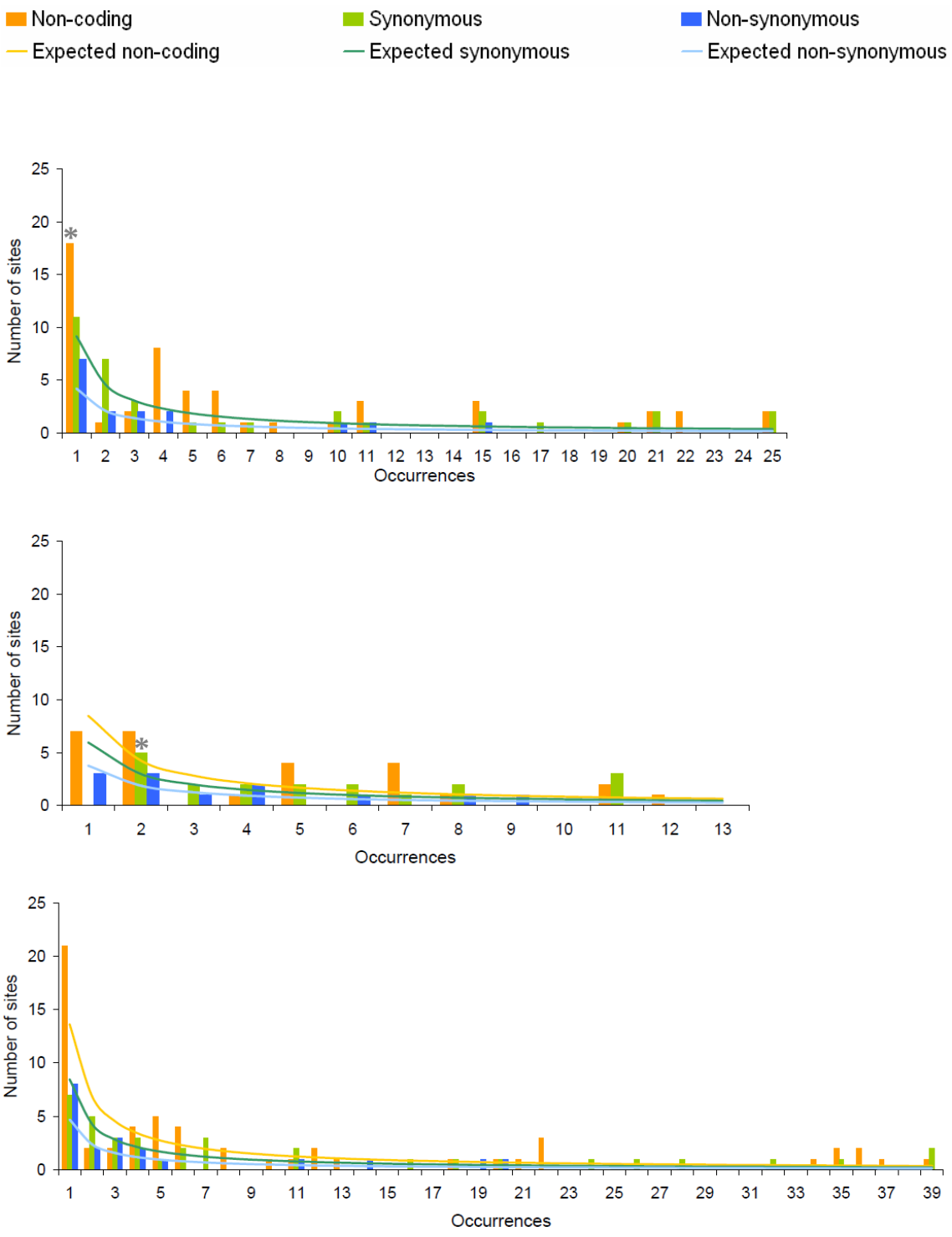


Fig. 7.—Frequency spectra of *Or33b* gene in African (A), non-African (B) populations, and the combination of African and non-African populations (C) of *D. melanogaster*. Gray star, $0.01 < P < 0.05$.

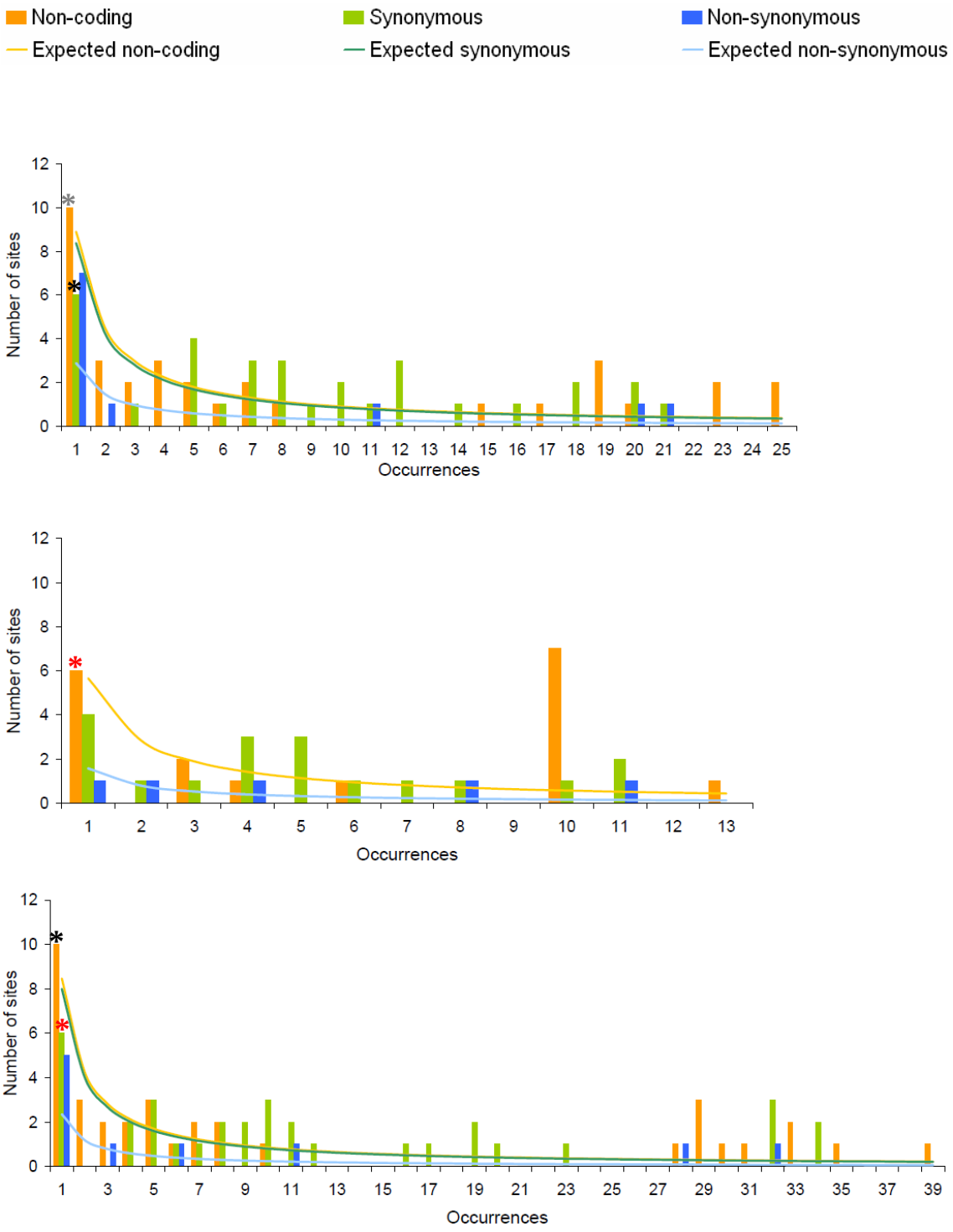


Fig. 8. —Frequency spectra of *Or33c* gene in African (A), non-African (B) populations, and the combination of African and non-African populations (C) of *D. melanogaster*. Gray star, $0.01 < P < 0.05$; black star, $0.001 < P < 0.01$; red star, $P < 0.001$.

Tests of neutrality

Tests of neutrality based on polymorphism data were applied to the *Or33a*, the intergenic region of *Or33a* and *Or33b*, *Or33b*, intergenic region of *Or33b* and *Or33c*, and *Or33c*. Tajima's *D* test, Fu and Li's *D** and *F** tests of *Or33a*, *Or33b* and intergenic region between *Or33b* and *Or33c* showed higher values in the non-African population than in the African population. Neither the Tajima's *D* test nor the Fu and Li' *D** and *F** tests detected any departure from neutral expectations in any populations. Fay and Wu's *H* test showed a significant degree of deviation from the neutral expectation in *Or33a* (African: $H = -1.466$; non-African: $H = -1.022$) and in the intergenic region between *Or33b* and *Or33c* (African: $H = -1.164$; $H = -1.386$) in *D. melanogaster* (table 5).

Table 5

Site frequency spectrum statistics for *Or33* genes

Gene	Length	Tajima's *D*	Fu and Li's *D**	Fu and Li's *F**	Fay and Wu's *H*
African (N = 26)					
Or33a	661	-0.378 (0.286)	-0.046 (0.462)	-0.176 (0.390)	**-1.466 (0.021)**
Intergenic region	494	-0.113 (0.450)	-0.085 (0.417)	-0.111 (0.425)	-0.269 (0.249)
Or33b	1371	-0.613 (0.132)	-0.948 (0.069)	-0.989 (0.071)	-0.298 (0.205)
Intergenic region	482	-0.318 (0.315)	-0.504 (0.227)	-0.523 (0.245)	**-1.164 (0.030)**
Or33c	1177	0.646 (0.956)	0.031 (0.510)	0.268 (0.698)	-0.075 (0.385)
Non-African (N = 14)					
Or33a	661	0.054 (0.541)	0.233 (0.601)	0.212 (0.589)	-1.022 (0.095)
Intergenic region	494	-0.594 (0.270)	-0.052 (0.454)	-0.228 (0.396)	0.096 (0.420)
Or33b	1371	1.088 (0.988)	1.274 (0.999)	1.405 (0.998)	0.121 (0.532)
Intergenic region	482	0.347 (0.693)	0.104 (0.529)	0.195 (0.564)	**-1.386 (0.047)**
Or33c	1177	0.697 (0.946)	0.500 (0.848)	0.636 (0.890)	-0.058 (0.412)

Table 5

(Continued)

Gene	Length	Tajima's *D*	Fu and Li's *D*	Fu and Li's *F*	Fay and Wu's *H*
African and non-African (N = 40)					
Or33a	661	-0.721 (0.133)	-0.436 (0.268)	-0.633 (0.204)	**-1.582 (0.012)**
Intergenic region	494	-0.649 (0.203)	-0.614 (0.218)	-0.744 (0.185)	-0.629 (0.117)
Or33b	1371	-0.419 (0.203)	-0.796 (0.106)	-0.789 (0.104)	-0.229 (0.256)
Intergenic region	482	-0.504 (0.237)	-0.777 (0.150)	-0.810 (0.152)	**-1.020 (0.036)**
Or33c	1177	0.511 (0.890)	0.031 (0.499)	0.237 (0.662)	-0.224 (0.273)

Note. —*P* values determined from 5000 coalescent simulations with recombination are shown in parenthesis. Significant *P* values (less than 5%) are shown in bold.

Discussion

Expression pattern of *Or33c*

The sexually dimorphic expression pattern in *Or33c* might be in connection with the hypothesis that may be involved in the detection of sex-specific ligand, such as pheromone (Goldman et al. 2005). And it can be seen as well that this pattern is shown in both the African and the non-African populations. On the other hand, in both sexes there is no significant difference in the expression level between populations, indicating that even function of *Or33c* may diverge in different populations due to different environmental constraints, it may not necessarily be accompanied with changes in sensitivity (which is correlated with expression level). Nevertheless, further functional analyses are needed to answer whether the driving force of positive selection of *Or33c* is related to the potential role on the detection of CH pheromones.

Demographic effect

As mentioned in the introduction, *D. melanogaster* populations have undergone complicated dispersal history, and this factor should leave footprints on the genome sequences. The lower nucleotide diversity and the higher level of LD in non-African population are consistent with the dispersal history leading to demographic effect of the non-African population. The non-African population is composed of samples from

worldwide localities. As a consequence, some linkage disequilibrium actually reflects geographical divergence, instead of true functional/physical correlations. Furthermore, samples from the populations which underwent bottleneck phase (e.g. American populations) would incorporate more fixation by genetic drift, thus cause more bias on LD data.

Signature of selection

It has been shown that *Or33c* may be acted on by positive selection in the lineage leading to ecologically diverse species of *Drosophila* (Tunstall et al. 2007). My study further characterizes the signature of positive selection of *Or33c* in *D. melanogaster* populations by McDonald and Kreitman test. In contrast to *Or33c*, neither of its tandem duplicate genes, *Or33a* and *Or33b* (Robertson, Warr, and Carlson 2003), shows a significant signature of positive selection. These patterns can be observed in both African and non-African populations. Both *Or33a* and *Or33b* are expressed in antenna, whereas *Or33c* is expressed in the maxillary palp (Vosshall, Wong, and Axel 2000). The acquisition of the Dyad-1 motif, which is required for the gene expression in the maxillary palp (Ray et al. 2007), in the 5' upstream region of *Or33c* might explain the expression pattern different from its duplicate genes. Furthermore, it has been shown that the upstream regulatory regions of *Or33c* and its co-expresser *Or85e* contain two

shared *cis*-regulatory motifs that are not present in the regulatory regions of any other Or genes (Ray et al. 2007). The acquired *cis*-regulatory motifs might involve the expression shift to different sensory organs and/or positive selection. This pattern is similar to *Or59b* and *Or59c*, which are also closely related tandemly duplicates (Robertson, Warr, and Carlson 2003). Although *Or59b* and *Or59c* have very similar sequence to each other, they have no shared regulatory motifs and their expression are different (*Or59b* expressed on antenna and *Or59c* expressed on maxillary palp) (Vosshall, Wong, and Axel 2000; Ray et al. 2007).

Mode of selection

A trend could be noticed that, values of Tajima's *D*, Fu and Li's *D** and *F** tests of *Or33a*, *Or33b* and intergenic region between *Or33b* and *Or33c* are larger, though not significant, in the non-African population than in the African one. This pattern may reflect the fact that the non-African population has experienced a population subdivision phase (Simonsen, Churchill, and Aquadro 1995), which is consistent with previous studies (Schlötterer, Vogl, and Tautz 1997).

Another question is, if there exists positive selection acting on *Or33c* as suggested by McDonald and Kreitman test, why don't we find the similar signature in other neutrality tests such as Tajima's *D*, Fu and Li's *D** and *F** tests? The difference

between these two kinds of tests is due to that McDonald and Kreitman test is based on the comparison of R/S ratios between divergence in two species and polymorphism within species, while other neutrality tests I applied in this study are based on the frequency spectra of polymorphic sites. If selection took action only during a period in the past, it could lead to an excess of fixation but no significant deviation from neutrality in polymorphism data. This mode of selection suggests that an episodic positive selection has been occurred between *D. melanogaster* and *D. simulans* lineages.

Hitchhiking effect in *Or33* gene family

The Fay and Wu's test could be used to infer whether the neutral locus in question is hitchhiked by a linked locus that is under positive selection (Fay and Wu 2000). It can be seen that in both the African and the non-African populations, intergenic region upstream to *Or33c* show a significant derivation from neutrality by Fay and Wu's test, suggesting that 5' upstream region of *Or33c* may be hitchhiked by *Or33c*. By further identified putative transcription factor binding site in the 5' upstream region of *Or33c* may help us to find evidence for this hypothesis.

On the other hand, although *Or33a* showed no sign of deviation from neutrality by the McDonald and Kreitman test, a significant deviation from neutrality had been

detected in the synonymous sites frequency spectra and in the Fay and Wu's test. This result suggests that this region of *Or33a* may be hitchhiked by the nearly narrow region.

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Appendix

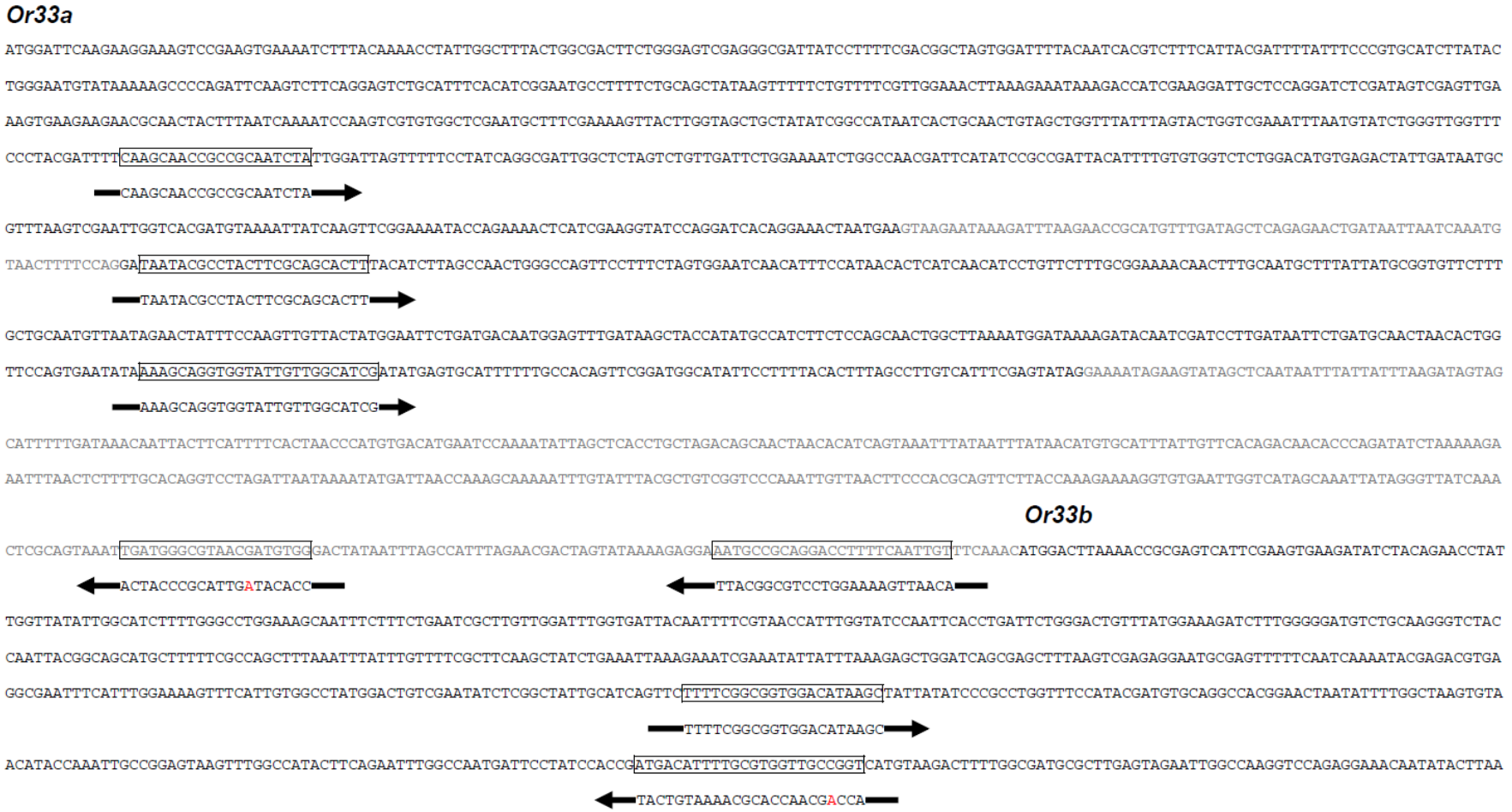

Fig. S1.—Location of PCR primers. Red indicates the modified nucleotides in primers.

Fig. S1.—(Continued)