Colonization of America by *Drosophila subobscura*: Association between *Odh* gene haplotypes, lethal genes and chromosomal arrangements

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The colonization of America by *Drosophila subobscura* has been a unique experiment in nature that has allowed us to explore the effects of evolution on a continental scale. To analyze this evolutionary event, nucleotide sequences of the *Odh* (Octanol dehydrogenase) gene were obtained for 43 lethal chromosomal lines from colonizing populations of North America and 5 from South America, in addition to 5 chromosomal lines from Europe with different viabilities and 2 from laboratory marker stocks. Since 10 different *Odh* haplotypes were found in America, the minimum number of colonizers would be 5 (or 3 mated females). Only one *Odh* haplotype was found in American O₅ inversions confirming that only one copy of this inversion was included among the sample of colonizers. The same *Odh* haplotypes were detected in association with the same chromosomal arrangements and with identical lethal genes in both North and South America indicating that exactly the same chromosome types reached both hemispheres. These observations indicate that the two continental colonizations are not independent. They are derived from the same colonization event. The population from which the colonization started should contain the O₅ inversion, a non-negligible frequency of the O₃₊₄₊₇ arrangement and all other arrangements found in America. So far the only populations that fulfill all these requirements are those from Greece, indicating that these populations can be considered good candidates as a starting point for an in depth analysis of the origin of the American colonization by *D. subobscura*.

Key words: chromosomal arrangements, colonization, *Drosophila subobscura*, lethal genes, *Odh*

INTRODUCTION

It is rare to have the opportunity to study the evolutionary consequences of recent colonization events. *Drosophila subobscura* previously considered to be a species with a Palearctic distribution, was first detected in Puerto Montt (Chile) in 1978 (Brncic et al. 1981). It spread rapidly throughout Chile and can now be found from La Serena to Punta Arenas (Prevosti et al. 1989). In 1982 *D. subobscura* was also found in Port Townsend (Washington, USA) (Beckenbach and Prevosti 1986). Its present distribution include the Western states of the USA and Canada from Port Hardy (British Columbia, Canada) (Huey et al. 2000) to Ojai (California) (Prevosti

et al. 1989). This double colonization is a grand experiment of evolution with two replicates (Ayala et al. 1989) that has allowed the predictable character of evolution and its speed to be analyzed. Analysis of chromosomal polymorphism has shown the rapid appearance of latitudinal clines in both North and South America that converge on those detected in the Old World (Prevosti et al. 1985; 1988; 1990). Clinal variation of other traits also converges on the pattern found in Old World flies. For example, although no wing-size cline was evident in North or South America one decade after the colonization (Pegueroles et al. 1995), after two decades a significant cline was evident in North American populations (Huey et al. 2000; Gilchrist et al. 2001).

Among the different genetic levels analyzed (Prevosti et al. 1983; Latorre et al. 1986; Rozas et al. 1990; Rozas and Aguadé 1991; Balaña et al. 1994; Pegueroles et al. 1996;

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1999; Noor et al. 2000; Pascual et al. 2001), one that has provided very valuable information on the colonization event has been that of lethal genes. These genes can only be studied for the O chromosome of *D. subobscura*, because this is the only one for which a balanced-lethal strain exists: *Va/Ba* (*Varicose/Bare*) (Sperlich et al. 1977). In American populations lethal genes do not distribute at random among the different chromosomal arrangements (Mestres and Serra 1999), in some cases being significantly associated with particular arrangements. This is the case for the complete association observed between the O₅ inversion and a lethal gene and for the partial association between the O₃₊₄₊₇ chromosomal arrangement and another lethal gene (Mestres et al. 1990; 1992; 1995; Solé et al. 2000). These associations were found in both North and South American populations suggesting that the colonizations were not independent (Mestres et al. 1992). Furthermore, the lethal genes have persisted in time, revealing a possible heterotic effect of these inversions (Mestres et al. 2001). As the O₅ inversion is not very common and has a rather erratic distribution in the Old World (Loukas and Krimbas 1980; Krimbas, 1993; Mestres et al. 1994; Zivanovic and Mestres 2000), it is likely that only one O₅ inversion (carrying the lethal gene) was included in the initial sample of colonizers (Mestres and Serra 1995). The significant latitudinal clines of the frequency of this inversion observed in colonizing populations (Prevosti et al. 1985; 1988; 1990) together with its complete association with a lethal gene (Mestres et al. 1990; 1992; 1995, Solé et al. 2000) and its significant persistence in America (Mestres et al. 2001), make it especially suitable to study not only the origin of the colonization, but also some of the evolutionary consequences of this event.

As suggested by the distribution of genes in *D. melanogaster* (Lindsley and Zimm 1992) we deduced that the *Odh* (Octanol dehydrogenase) gene could be located inside and very close to the proximal breakpoint of the O₅ inversion. Its location in *D. subobscura* has been confirmed in the present research using the *in situ* hybridization technique. Nucleotide sequences for *Odh* were obtained for different O₅ chromosomal lines from Europe and America, as well as in other lines with different chromosomal arrangements in order to analyze the relationship between the haplotypes and inversions. To date seven different arrangements have been detected in American populations (O_{st}, O₅, O₇, O₃₊₄, O₃₊₄₊₂, O₃₊₄₊₇ and O₃₊₄₊₈) (Prevosti et al. 1985; 1988; 1990). The O chromosome is usually divided into two regions: Segment I (about 1/3 of the total O chromosome length) covers the area in which arrangement O₃₊₄ is located and segment II (about 2/3 of the whole chromosome) corresponds to the remainder of the chromosome (Krimbas 1993). The *Odh* gene is located in segment II, and is included in the O₇ and O₅ inversions. This gene is close (5 chromosomal

sections) to the proximal point of the O₂ inversion. In all other situations the *Odh* gene could freely recombine. The rich chromosomal polymorphism of *D. subobscura* and the founder event of the American colonization could generate different kinds of associations. Associations between molecular markers and inversions have been studied in different species of the genus *Drosophila* (for a complete review see Andolfatto et al. 2001), including *D. subobscura* (Rozas and Aguadé 1994; Rozas et al. 1999; Munté et al. 2000).

In addition to the adaptive changes that have taken place in the American populations since the beginning of the colonization (Prevosti et al. 1985; 1988; 1990; Huey et al. 2000; Gilchrist et al. 2001), there are some basic questions which demand explanations. One of them is to estimate the initial number of colonizers, and to ascertain which Palearctic population colonizers came from. Another related topic is the attempt to track the chromosomes or chromosomal fragments that were included within the initial sample of flies. Finally, an in depth study of the genetic relationship between North and South American populations has to be carried out. All these basic questions can be approached with the information obtained in this work using the nucleotide haplotypes of the *Odh* gene and the lethal genes as genetic markers of the chromosomal inversions, with which they are associated.

MATERIALS AND METHODS

Chromosomal lines sequenced All chromosomal lines with their arrangements in which the *Odh* gene has been sequenced are described in Table 1. Most of them are lethal chromosomal lines from three North American localities: Gilroy (California), Bellingham and Centralia (Washington). They were obtained with appropriate genetic crosses using the balanced-lethal strain *Va/Ba*. These crosses have been described in detail elsewhere (Mestres et al. 1990; 1992; 1995). The Gilroy population was sampled in two different periods denoted as Gilroy I (1984 – 1985) (Mestres et al. 1990) and Gilroy II (1990 – 1991) (Mestres et al. 1992). Eleven lethal chromosomal lines were sequenced in Gilroy I and 8 in Gilroy II. The Bellingham collection was obtained in 1991 (Mestres et al. 1995) and 13 lethal chromosomal lines have been analyzed. The Centralia population was sampled in 1995 (Solé et al. 2000) and 11 lethal chromosomal lines have been sequenced. Furthermore, two lethal chromosomal lines (both carrying the O₅ inversion) from the Chilean population of Puerto Montt (Mestres et al. 1990) and 3 from Santiago de Chile (Mestres et al. 1992) were analyzed. Although it is difficult to find the O₅ inversion in European populations, some O₅ chromosomal lines were sequenced: T15, a lethal line from the French population of Taulé (Mestres et al. 1992); L25, a semilethal

Table 1. Description of all chromosomal lines that have been sequenced for the *Odh* gene.

Chromosomal line	Arrangement	Viability	Population	Country	Accession number
G7A	O ₅	LCL	Gilroy I	U.S.A.	AJ496667
G32	O ₃₊₄₊₂	LCL	Gilroy I	U.S.A.	AJ496668
G34	O ₃₊₄₊₂	LCL	Gilroy I	U.S.A.	AJ496669
G39	O ₃₊₄₊₇	LCL	Gilroy I	U.S.A.	AJ496670
G41	O ₃₊₄₊₇	LCL	Gilroy I	U.S.A.	AJ496671
G61	O ₅	LCL	Gilroy I	U.S.A.	AJ496672
G66	O ₃₊₄₊₂	LCL	Gilroy I	U.S.A.	AJ496673
G209	O ₃₊₄₊₇	LCL	Gilroy I	U.S.A.	AJ496674
G215	O ₃₊₄	LCL	Gilroy I	U.S.A.	AJ496675
G226	O ₅	LCL	Gilroy I	U.S.A.	AJ496676
G240	O ₅	LCL	Gilroy I	U.S.A.	AJ496677
FGF5	O ₃₊₄₊₇	LCL	Gilroy II	U.S.A.	AJ496678
GM4	O ₅	LCL	Gilroy II	U.S.A.	AJ496679
GM24	O ₃₊₄₊₂	LCL	Gilroy II	U.S.A.	AJ496680
GM107	O ₃₊₄₊₂	LCL	Gilroy II	U.S.A.	AJ496681
GM111	O ₃₊₄₊₇	LCL	Gilroy II	U.S.A.	AJ496682
GM121	O ₃₊₄₊₇	LCL	Gilroy II	U.S.A.	AJ496683
GF3I	O ₃₊₄₊₂	LCL	Gilroy II	U.S.A.	AJ496684
GM1BK	O ₃₊₄₊₇	LCL	Gilroy II	U.S.A.	AJ496685
BM1	O _{st}	LCL	Bellingham	U.S.A.	AJ496686
BM2	O ₅	LCL	Bellingham	U.S.A.	AJ496687
BM5	O _{st}	LCL	Bellingham	U.S.A.	AJ496688
BM41	O ₃₊₄₊₈	LCL	Bellingham	U.S.A.	AJ496689
BM42	O ₅	LCL	Bellingham	U.S.A.	AJ496690
BM63	O ₅	LCL	Bellingham	U.S.A.	AJ496691
BM79	O ₅	LCL	Bellingham	U.S.A.	AJ496692
BM90	O ₅	LCL	Bellingham	U.S.A.	AJ496693
BF8	O ₅	LCL	Bellingham	U.S.A.	AJ496694
BF20	O _{st}	LCL	Bellingham	U.S.A.	AJ496695
BF30	O ₅	LCL	Bellingham	U.S.A.	AJ496696
BF66	O ₃₊₄₊₂	LCL	Bellingham	U.S.A.	AJ496697
BF69	O _{st}	LCL	Bellingham	U.S.A.	AJ496698
C6B	O ₃₊₄	LCL	Centralia	U.S.A.	AJ496699
C21H	O ₃₊₄₊₈	LCL	Centralia	U.S.A.	AJ496700
C22A	O ₅	LCL	Centralia	U.S.A.	AJ496701
C57B	O ₅	LCL	Centralia	U.S.A.	AJ496702
C61E	O ₅	LCL	Centralia	U.S.A.	AJ496703
C66D	O ₃₊₄₊₈	LCL	Centralia	U.S.A.	AJ496704
C93B	O ₃₊₄₊₈	LCL	Centralia	U.S.A.	AJ496705
C94B	O ₃₊₄₊₈	LCL	Centralia	U.S.A.	AJ496706
C105J	O ₅	LCL	Centralia	U.S.A.	AJ496707
C110N	O ₅	LCL	Centralia	U.S.A.	AJ496708
C185I	O ₃₊₄₊₈	LCL	Centralia	U.S.A.	AJ496709
P282	O ₅	LCL	Puerto Montt	Chile	AJ496710
P269	O ₅	LCL	Puerto Montt	Chile	AJ496711
S16	O ₃₊₄₊₂	LCL	Santiago de Chile	Chile	AJ496712
S47	O ₅	LCL	Santiago de Chile	Chile	AJ496713
S49	O ₃₊₄₊₇	LCL	Santiago de Chile	Chile	AJ496714
T15	O ₅	LCL	Taulé	France	AJ496715
L25	O ₅	SLCL	Lilla-Edet	Sweden	AJ496716
LNF21	O ₅	NCL	Louvain-la-Neuve	Belgium	AJ496717
C31	O ₃₊₄₊₈₊₇	LCL	Bordils	Spain	AJ496718
F122	O ₃₊₄₊₂₂	NCL	Observatori Fabra	Spain	AJ496719
chcu	O ₃₊₄	HCL	Laboratory strain	—	AJ496720
Va	O _{3+4+VIII+210}	BLC	Laboratory strain	—	AJ496721

LCL stands for “lethal chromosomal line”, SLCL “semilethal chromosomal lines”, NCL “normal chromosomal line”, HCL “homokaryotypic chromosomal line” and BLC “balancer lethal chromosome”.

line from the Swedish population of Lilla Edet (Mestres et al. 1992); and LNF21, a line with normal viability and maintained in homokaryotypic O_5/O_5 condition from the Belgian population of Louvain-la-Neuve, obtained from a 1999 sample.

Finally, we studied the F122 homokaryotypic chromosomal line (O_{4+3+22}/O_{3+4+22}). The O_{22} inversion has very similar breakpoints to those of the O_5 . It was obtained in a collection from Observatori Fabra (Barcelona) (Mestres et al. 1998). A lethal chromosomal line (C31) with the very infrequent arrangement $O_{3+4+8+7}$ from the Bordils population (about 90 km NE of Barcelona) (Mestres et al. 1990) was also sequenced. In addition, the sequences of the *Odh* gene of the laboratory stock chromosomes *Va* and *chcu* were obtained.

In situ hybridization on polytene chromosomes

Polytene chromosome squashes were prepared from *D. subobscura* individuals of the homokaryotypic (O_{3+4}/O_{3+4}) *chculchcu* (*cherry curled*) strain using the procedure described by Visa et al. (1991). The DNA probe was obtained from *D. melanogaster* (Luque et al. 1994) and was labeled with digoxigenin-dUTP (Roche Diagnostics nick translation kit). Hybridization and detection were carried out using the procedures described by de Frutos et al. (1990), with reagents from the digoxigenin-nucleic acid detection kit (Roche Diagnostics).

Strains and DNA preparation Isolation of total DNA from a single individual was carried out according to the method of Latorre et al. (1986).

PCR cloning and sequencing Two sets of degenerate primers (C1-CD6 and C2-CD4) were designed based on the sequence of *D. melanogaster Odh* (accession number U07641). The conserved regions for the design of the primers were chosen based on the alignment of amino acid sequences of this gene from several species (Luque et al. 1994) (Fig. 1).

PCR reactions were performed using the GeneAmp 2400 System (Perkin Elmer). Cycling conditions were 94°C for 5 min; 35 cycles of 94°C for 10 s, 55°C for 30 s, 72°C for 1 min; and a final extension of 4 min at 72°C. PCR reactions contained 200mM dNTPs (AP Biotech), 20 pmol of each set of primers, 1x Taq buffer, 1.5 u of Taq polymerase (AP Biotech) and 10 to 40 ng of template in a final volume of 50 µl.

The PCR fragments (264 bp for C1-CD6 and 800 bp for C2-CD4) of several strains were excised from a 1.4% agarose gel in 0.5xTBE and extracted using the GFX™ PCR DNA and Gel Band Purification kit (AP Biotech). PCR products were cloned into T-pBluescript sk(+) (Stratagene), prepared as in Marchuk et al. (1992) and transformed into *E. coli* XL1-Blue MRF™ (Stratagene). Plasmid DNA extractions were carried out using the CONCERT™ Rapid miniprep system (Gibco-BRL). Sequencing reactions were performed in a PE/ABI 373 and 377 at the Servei de Seqüenciació of the Universitat de València, using the dRhodamine ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and two primers (T7 and T3 pBluescript).

The sequences of these cloned fragments were used to design new internal primers (ODH-F, ODH-R, ODHseq-F

Fig. 1. Diagrammatic representation of the *Odh* gene fragment of *D. subobscura*. Arrows indicate the relative position of the primers. Intron 3 is not present in *D. melanogaster* (dotted box). Black line indicates the sequenced fragment, 793 nucleotides long. Nucleotides: exon 2, 60; intron 2, 65; exon 3, 462; intron 3, 51; exon 4, 155.

and ODHseq-R, see Fig. 1) specific for *D. subobscura* *Odh*.

The procedure implies a first PCR with C1 and CD4 (PCR conditions as described above), followed by a nested PCR with ODH-F and ODH-R, using 1 µl of first PCR as template for nested PCR. Cycling conditions for nested PCR were 94°C for 3 min; 5 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min; 35 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min; and a final extension of 4 min at 72°C. This PCR product was ethanol precipitated and direct sequenced using the internal primers C2, CD6, ODHseq-F and ODHseq-R (Fig. 1). *Odh* genes were sequenced on an ABI PRISM™ 377 (Perkin Elmer) of the Servei de Seqüenciació Automàtica of the Universitat de Barcelona.

Sequence alignment and phylogenetic analysis

Consensus sequence identification and assemblage of all readings of each clone was performed by analysis of the electropherograms with SEQUENCHER v3.0 (Genecodes co.). Sequence alignments were performed with BioEdit v. 4.8.6 (Hall 1999; <http://www.mbio.ncsu.edu/BioEdit>). DNA polymorphism was analyzed using DnaSP version 3 (Rozas and Rozas, 1999). The Kimura 2-parameter distance model and the Neighbor-Joining (NJ) tree reconstruction algorithm (Saitou and Nei 1987) were conducted using MEGA v. 2 (Kumar et al. 2001).

RESULTS

In situ hybridization Analysis of the *in situ* hybridization showed that the *Odh* gene is located within section 84 B of the O chromosome of *D. subobscura* according to the Kunze-Mühl and Müller map (1958). It is located in the homologous position of the *D. melanogaster* *Odh* (Luque et al. 1994), inside and very close to the proximal breakpoint of the O₅ inversion. It is also located within the chromosomal inversions O₇ (found in American populations), O₁ and O₂₂ (both absent in America), but outside O₂ (a frequent inversion in America), close to its proximal breakpoint.

Analysis of the sequences 55 sequences of a 793 nucleotides fragment of *Odh* were obtained. This fragment contains part of exon 2, intron 2, exon 3, intron 3 and part of exon 4. Intron 3, 51 nucleotides long, is present in *D. subobscura*, but not in *D. melanogaster*. This intron, predicted by Southern blot (Luque et al. 1994) has been confirmed in the present research. The distribution of this intron in different evolutionary lineages is described by Canestro et al. 2001.

One of the main problems in the analysis of the sequences was to the fact that most of the chromosomal lines (51) were heterokaryotypic, carrying one *Va* balancer chromosome and one wild type chromosome. Thus, two *Odh* alleles (one from each chromosome) were ampli-

fied and sequenced together. It was essential to distinguish between the *Odh* allele of the *Va* chromosome and that of wild type. Fortunately, some individual *Odh* sequences were obtained by cloning in plasmids during the process of primers design. At least one of them was expected to belong to the *Odh* gene of the *Va* chromosome. Clear polymorphic sites were often detected in the chromatograms, in which one nucleotide belonged to the *Odh* allele of the *Va* chromosome. Comparing these positions with the individual sequences it was possible to identify the *Odh* allele of the *Va* balancer chromosome and use it as reference in all analyses. No polymorphisms due to insertion/deletion were found, in either the exons or the introns.

Odh haplotypes in different chromosomal arrangements

The pattern of polymorphisms of the *Odh* gene in different chromosomal arrangements is shown in Fig. 2. All O₅ inversions from North and South America have the same *Odh* haplotype which confirms that only one of these inversions was carried by the sample of colonizers (Mestres and Serra 1995). The O₅ inversions from Taulé (France) and Lilla-Edet (Sweden) have the same *Odh* haplotype as that found in America but the haplotype of the O₅ inversion from Louvain-la-Neuve (Belgium) has four different substitutions. Nine lethal chromosomal lines with the O₃₊₄₊₂ arrangement were sequenced, all of them presenting the same haplotype. The *Odh* gene is outside but close (about 8 chromosomal sections) to the proximal breakpoint of the O₂ inversion. All these lines are lethal and out of 36 crossings performed some of them proved to be allelic (Mestres et al. 1992; 1995). Seven lines with the O₃₊₄₊₇ arrangement (all carrying the same lethal gene) were sequenced. The *Odh* gene is located inside the O₇ inversion. In general, the haplotypes found in this arrangement have few polymorphism than those found in other arrangements. Two groups can be clearly distinguished: one corresponding to the haplotype found in chromosomal lines GM1BK, S49 and G209; the other corresponding to the haplotype found in chromosomal lines GM111, GM121, G39 and FGF5, which have the same non-synonymous substitution (position 509). The sequence of FGF5 differs from the others (GM111, GM121 and G39) by one substitution (position 167). All sequences analyzed in O₃₊₄₊₇ chromosomal lines are likely to have a common origin because they lack some substitutions in the *D. subobscura* specific intron that are found in all other sequenced lines. These seven O₃₊₄₊₇ chromosomal lines have the same lethal gene. Four different haplotypes were observed in the O₃₊₄₊₈ arrangement (located far away from the *Odh* gene). One of them (detected in the C185I line) is identical to that observed in the O₃₊₄₊₂ chromosomes. The C21H line has a haplotype identical to one detected in three O_{st} lines. Finally, two more haplotypes were observed in the O₃₊₄₊₈ arrange-

	Exon 2										Exon 3										Intron <i>D. subobscura</i>										Exon 4									
	20	87	102	103	119	167	213	221	254	272	305	311	329	374	455	479	491	509	510	557	572	575	602	604	609	610	614	616	628	644	655	680	683	686						
Va	T	C	G	T	T	C	G	C	C	C	T	G	C	C	C	G	G	G	A	G	C	A	G	T	C	G	T	C	A	G	C	A	T	A						
chcu											A				A				T				A										C							
BF 20 _{st}	A	G					T				A		G	T	A	A			T	C			A	T	A	G			C	A		T	C	T						
C66D ₃₊₄₊₈	A	G					T				A		G	T	A	A			T	C			A	T	A	G			C	A		T	C	T						
C93B ₃₊₄₊₈	A	G					T				A		G	T	A	A			T	C			A	T	A	G			C	A		T	C	T						
C6B ₃₊₄	A	G					A	T			A		G	T	A	A			T	C			A	T	A	G			C	A		T	C	T						
G215 ₃₊₄					C		A				A	A			A				T			G	T	A	T	A	G		C	A		T	C	T						
BM5 _{st}					C						A	A			A				T			G	T	A	T	A	G		C	A		T	C	T						
BM1 _{st}					C						A	A			A				T			G	T	A	T	A	G		C	A		T	C	T						
BF69 _{st}					C						A	A			A				T			G	T	A	T	A	G		C	A		T	C	T						
C21H ₃₊₄₊₈					C						A	A			A				T			G	T	A	T	A	G		C	A		T	C	T						
GM1BK ₃₊₄₊₇								T			A				A				T			A		A									C							
S49 ₃₊₄₊₇								T			A				A				T			A		A									C							
G209 ₃₊₄₊₇								T			A				A				T			A		A									C							
GM111 ₃₊₄₊₇											A				A	A	T	T	T				A										C							
GM121 ₃₊₄₊₇											A				A	A	T	T	T				A										C							
G39 ₃₊₄₊₇											A				A	A	T	T	T				A										C							
FGF5 ₃₊₄₊₇											A				A	A	T	T	T				A										C							
C31 ₃₊₄₊₇₊₈											A				A				T	C	A		A										C							
BM41 ₃₊₄₊₈											A				A	A			T	C			A										C							
C94B2 ₃₊₄₊₈											A				A	A			T	C			A										C							
C185 ₃₊₄₊₈											A				A								G	T	A	T	A	G		C	A		T	C	T					
GM24 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
GF31 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
G66 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
GM107 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
S16 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
BF66 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
G32 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
G34 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
G41 ₃₊₄₊₂											A				A				T				G	T	A	T	A	G		C	A		T	C	T					
F122 ₃₊₄₊₂₂											A				A								G		T	T	G		C	A		T		T						
BF30 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
BF8 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
BM2 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
BM42 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
BM63 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
BM79 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
BM90 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
C57B2 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
C22A ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
C105J ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
C110N ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
C61E ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
GM4 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
G7A ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
G61 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
G240 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
G226 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
P282 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
P269 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
S47 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
T15 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
L25 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						
LNF21 ₅											A						C	C	T	C			A	T	A	G			C	A		T	C	T						

Fig. 2. Nucleotide polymorphisms detected in all chromosomal lines sequenced for the *Odh* gene, using the Va allele as reference.

ments, one in the C66D and C93B lines and the other in the BM41 and C94B2 lines. Only two lines with the O_{3+4} arrangement were sequenced. In this case the *Odh* gene is also located far away from the breakpoint of this arrangement. The two sequences are very different from each other, but the C6B line resembles lines C93B, C66D (both O_{3+4+8}) and BF20 (O_{st}), and line G215 is very similar to lines BM5, BM1, BF29 (all O_{st}) and C21H (O_{3+4+8}). Four O_{st} chromosomal lines were sequenced, showing two allelic forms: one is detected in lines BM1, BM5 and BF69, being identical to that of line C21H (O_{3+4+8}); the other (BF20) is identical to that of C66D and C93B (both O_{3+4+8}). The location of *Odh* in chromosomal lines O_{3+4} , O_{3+4+8} and O_{st} allows the possibility of recombination and other forms of genetic exchange. The F122 line (O_{3+4+22}) shows a very different pattern of polymorphism to that of the O_5 lines, although they have similar breakpoints (Kunze-Mühl and Müller 1958; Mestres et al. 1998), demonstrating an independent origin of the two inversions. Finally, the C31 line (carrying the uncommon arrangement $O_{3+4+8+7}$) has a special haplotype. It resembles the

O_{3+4+7} haplotypes more than those of O_{3+4+8} , probably due to the position of *Odh* (included in the O_7 inversion).

Table 2. Estimates of nucleotide polymorphism of *Odh* gene within different groups of arrangements. n = number of sequences; $Nhap$ = number of haplotypes; h = haplotype diversity; S = number of polymorphic sites; π = nucleotide diversity; θ = expected average number of nucleotide differences; k = average number of nucleotide differences.

	O_5 and O_{3+4+7}		All other arrangements	
n	27		21	
$Nhap$	4		6	
$h \pm SD$	0.442	± 0.109	0.743	± 0.083
S	17		17	
$\pi \pm SD$	0.00706 $\pm$ 0.00160		0.00807 $\pm$ 0.00109	
$\theta \pm SD$ (per site)	0.00556 $\pm$ 0.00216		0.00596 $\pm$ 0.00240	
$k \pm SD$	5.595	± 2.772	6.400	± 3.158
$\theta \pm SD$ (per sequence)	4.411	± 1.712	4.725	± 1.903

Regarding the *Odh* gene itself, we also studied its nucleotide polymorphism for the American lethal chromosomal lines, grouping them into two sets: those in which the *Odh* gene is located within an inversion (O_5 and

O_{3+4+7}) and those in which it is not (all other chromosomal arrangements). The values of the parameters that describe this nucleotide polymorphism are shown in Table 2. The first group exhibits lower diversity than

Fig. 3. Neighbor-joining phylogenetic tree obtained using all the *Odh* nucleotide sequences. A clear association between the nucleotide sequences and chromosomal arrangements can be observed.

the second group (for haplotype diversity the SD ranges between the two groups do not overlap), probably due to the founder event and by the restriction of free interchange of genetic information.

A neighbor-joining phylogenetic tree was constructed to obtain an objective classification of the haplotypes according to their similarities (Fig. 3). A clear cluster is obtained for all *Odh* sequences of the O_5 inversion and for sequences in the arrangements O_{3+4+7} (including the sequences of the *Va* and *chcu* chromosomes and the line $O_{3+4+8+7}$) independently of their origin. The situation for the sequences in which the *Odh* gene is not included in any inversion (O_{3+4} , O_{3+4+2} , O_{3+4+8} and O_{st}) is less clear. Although all O_{3+4+2} are grouped together (as mentioned previously), *Odh* is outside of the inversion, but not far away (about 5 chromosomal sections) from the proximal breakpoint.

DISCUSSION

The similarity between North and South America

Our analysis of the *Odh* gene confirms the high degree of genetic resemblance between North and South American populations previously demonstrated in other studies (Prevosti et al. 1988; 1989; Balanyà et al. 1994; Rozas and Aguadé 1991; Mestres et al. 1990; 1992; 1995; Solé et al. 2000). All O_5 chromosomal lines have the same haplotype in both North and South America. Furthermore, other chromosomal lines carrying the same haplotype are also found in both continents: the Chilean line S49 (O_{3+4+7}) has the same haplotype as that observed in the Californian lines GM1BK and G209, which also carry the O_{3+4+7} arrangement (Fig. 2). The Chilean line S16 (carrying the O_{3+4+2} arrangement) has the same haplotype as that found in the O_{3+4+2} chromosomal lines from North America. Due to the founder event and the extraordinarily rich chromosomal polymorphism for inversions in this species, all levels of genetic variation (allozymes, lethal genes, nucleotide sequences) are usually associated to a greater or lesser degree with particular chromosomal arrangements in the American populations (Prevosti et al. 1983; 1989; Mestres et al. 1990; 1992; 1995; Mestres and Serra 1999), so the patterns of genetic variability observed will depend on the distribution of these inversions. Moreover, the lethal genes detected in these arrangements are allelic in North and South America. As mentioned above, American O_5 inversions have the same haplotype for the *Odh* gene and the same lethal gene (Mestres et al. 1990; 1992; 1995; Solé et al. 2000). The Chilean line S16 (O_{3+4+2}) and the North American lines carrying this arrangement have the same haplotype and some of these North American lines also have the same lethal gene found in the S16 line. A similar situation is observed in the Chilean line S49 (O_{3+4+7}); it has the same haplotype, chromosomal arrangement and lethal

gene as the Californian lines G209 and GM1BK. The same associations (chromosomal arrangements, lethal genes and *Odh* haplotypes) observed between North and South American populations suggest that at least some identical chromosomes carried by the sample of colonizers reached both hemispheres.

As both colonized areas are widely separated and there is no gene flow between them, it is most likely that in the first step of the colonization a rather small sample of colonizers reached one of the hemispheres. After that, a large sample of secondary colonizers should have reached the other hemisphere. There was no substantial loss of genetic variability in the second step, as revealed by the available genetic data (Prevosti et al. 1988; 1989; Mestres et al. 1992; Balanyà et al. 1994; Pascual et al. 2001). However, we still don't know which hemisphere was colonized first, or how the colonizers were carried to the colonized areas. It is also difficult to explain the fact that after 500 years of commercial contact between America and the Palearctic region *D. subobscura* has only recently reached the New World. Thus, we have a natural experiment with two replicates (North and South America), with a very similar initial genetic variability and almost equivalent pattern of climates. However, the evolutionary processes have not been identical in both colonized areas: the heterotic effect of the O_5 inversion is different in both hemispheres (Mestres et al. 2001) and the frequency clines of some chromosomal arrangements with latitude have not undergone the same evolutionary trajectories in both hemispheres (Balanyà et al. 2003). There are likely to be important differences in both hemispheres with regard to some environmental conditions (physical and biological), for instance, there are other species of the *obscura* group in North America, but not in South America.

The number of colonizers Knowledge of the initial number of colonizers is a major topic, because it could indicate the magnitude of the founder effect (Brncic et al. 1981; Prevosti et al. 1985; Mestres et al. 1990; Rozas and Aguadé 1991; Pascual et al. 2001). In our study, ten different haplotypes were observed for the *Odh* gene in the American lines. Thus, the minimum number of individuals necessary to "pack" all this genetic variation is 5 (or 3 mated females). This value is similar to that obtained by Rozas and Aguadé (1991) based on four-cutter restriction map analysis of the *rp49* gene. Furthermore, as only one haplotype has been detected in all American O_5 chromosomal inversions (which is completely associated with a lethal gene), the hypothesis that only one O_5 inversion was included in the initial sample of colonizers is confirmed (Mestres and Serra 1995). Thus, due to the founder effect, all O_5 inversions found in America are copies of the original inversion, which probably increased in frequency by genetic drift and natural selection (Mestres

et al. 2001).

Gathering together the information obtained from different procedures, estimates of the initial number of colonizers range between 10 and 150. Assuming a western Mediterranean origin the estimate using the chromosomal polymorphism gives an initial sample of 10 to 15 individuals (Brncic et al. 1981; Prevosti et al. 1985). With restriction map analysis, the number of O chromosomes for the *rp49* region that expanded in America was estimated as 8 to 12 (Rozas and Aguade 1991). Using microsatellite data, the estimate gives similar values (4 to 11 individuals; Pascual et al. 2001). The maximum range of colonizers was obtained by joint analysis of chromosomal inversions and lethal genes (10 to 150 individuals; Mestres et al. 1990).

The origin of the colonization The population from which the colonization started must have a genetic structure that fits with the different genetic traits found in the Americas. Specifically, it must contain all the chromosomal inversions and arrangements that are detected in the colonized areas (Prevosti et al. 1985; 1988; 1990), with the exception of those that have arisen there recently: namely E₁₇ (Brncic et al. 1982), E₁₈ (Pegueroles et al. 1988), E₂₁ and O₂₆ (Balanyà et al. 2003), and E₂₃ (Balanyà et al. (2004). The O₅ inversion, with a rather erratic distribution in the Palearctic region (Krimbas 1993; Zivanovic and Mestres 2000) is a good landmark for tracing the origin of the American populations. It has non-negligible frequencies in these populations and is distributed according to significant latitudinal clines (Prevosti et al. 1985; 1988; 1990). Furthermore, in spite of being completely associated with a lethal gene it has an heterotic effect (Mestres et al. 2001). A Palearctic population containing an O₅ inversion carrying a lethal gene allelic to that found in America and with the same *Odh* haplotype as that carried by the American O₅ inversions, would be a very good candidate for the population from which the colonization started. Palearctic O₅ inversions show variability for lethal genes (Mestres et al. 1992; Zivanovic and Mestres 2000) and for *Odh* nucleotide sequences. The O₅ haplotype found in America has also been observed in the O₅ inversions from Taulé (France) and Lilla-Edet (Sweden), but not in the O₅ inversion from Louvain-la-Neuve (Belgium) which indicates that there is more genetic variability within this arrangement in European populations. The viability and *Odh* haplotypes of more O₅ inversions should be analyzed in order to obtain more information about the population from which the colonization started. This is not an easy task, however, given its low frequency and its rather erratic distribution in the Palearctic area.

Three different *Odh* haplotypes have been observed in association with American O₃₊₄₊₇ arrangements. Since these arrangements were most probably included in the

sample of chromosomes carried by the colonizers, their frequency in the population of origin should be rather high. It is possible to analyze the distribution of O₅ and O₃₊₄₊₇ arrangements in the Palearctic region (Krimbas 1993) and compare the areas in which they overlap. With this information it has been possible to select those populations in which the O₅ inversion and also a non-negligible frequency of O₃₊₄₊₇ are found. The populations fulfilling this requirement and also having all other chromosomal arrangements found in America are: Mt. Parnes (Greece) when gathering together data from different years (Krimbas 1967; Krimbas and Alevizos 1973); Zürich (Switzerland), in a collection of 720 individuals (Burla and Götz 1965) and in a later collection of 942 individuals (Gosteli 1990); and finally in the Swiss canton of Valais when grouping together the sub-samples collected at different altitudes (Burla et al. 1986). Whilst the frequency of O₃₊₄₊₇ arrangement ranges between 2.40 and 6.65% in Mt. Parnes, in the Swiss populations this frequency is negligible (0.07 and 0.11% in Zürich and 0.8% in canton of Valais). Thus, Greece seems to be the most likely region from which the colonization could have started. Other evidence in favor of this hypothesis is provided by the analysis of the O₃₊₄₊₂ arrangement. Even though only one haplotype of the *Odh* gene has been detected in all O₃₊₄₊₂ chromosomal lines studied, in the early analysis some copies of this arrangement that are free of lethals and some carrying the same lethal gene were found in both North and South America. Thus, it is likely that one or at most two O₃₊₄₊₂ arrangements were included in the original sample of colonizers. Accordingly, the population of origin should contain this arrangement, probably with a frequency lower than that of the O₃₊₄₊₇ arrangement. Again, the population of Mt. Parnes fits all these requirements (Krimbas 1967). Finally, this hypothesized Mediterranean origin of the colonizers is consistent with the pattern of enhanced evolution in Northern latitudes observed by Gilchrist et al. (2001) when analyzing rates of evolution for quantitative traits in colonizing populations of this species.

These interpretations still present some difficulties. The population of Mt. Parnes exhibits a non-negligible frequency of the O₃₊₄₊₁ (19.84 – 28.00%) and O₃₊₄₊₂₂ (2.38 – 11.47%) chromosomal arrangements (Krimbas 1967) and these arrangements have never been found in America. A sample size of 100 individuals should statistically contain both arrangements. Furthermore, later analyses of chromosomal polymorphism in the Greek populations of Mt. Parnes (Sept. 1975 and April 1977), Crete (Sept. 1977) and Preveza (Sept. 1975) did not detect the O₅ inversion (Loukas et al. 1979; 1980; 1981). These samples were collected just before the first detection of *D. subobscura* in Puerto Montt (Chile). Mt. Parnes was not the only Greek population in which the O₅ inversion was detected. It was also present in the analyses of Athens,

Poros, Vytina and Cephalonia (Krimbas 1964). In some of them the frequency of O_{3+4+1} is lower than that recorded in Mt. Parnes and O_{3+4+22} is absent. Thus, the frequencies of the O chromosomal arrangements vary on a geographical scale when analyzing different Greek populations. Furthermore, there is evidence of microdifferentiation in one population: Krimbas and Alevizos (1973) found differences in chromosomal polymorphism when comparing different traps. For these reasons a small sample of colonizers from a Greek population (most probably 10 to 15 individuals, as suggested in the previous section) could contain an O_5 inversion without the O_{3+4+1} or O_{3+4+22} chromosomal arrangements.

Origin and persistence of the association between the *Od*h haplotypes and chromosomal arrangements Chromosomal arrangements are considered important substrates for adaptation during colonizing events (Lee 2002). Thus, it is important to recognize, using different genetic markers and their association with chromosomal arrangements, the chromosomes or chromosomal segments that were included in the initial sample of colonizers. The distribution of the *Od*h haplotypes in the American populations is mainly determined by the distribution of those chromosomal arrangements with which these haplotypes are. This nonrandom association between *Od*h haplotypes and some chromosomal arrangements can be explained both, by the chromosomal composition of the colonizing sample - in which some arrangements, such as the O_5 inversion, were represented only once - and by a lack of free genetic exchange in the O_5 , O_2 and O_7 inversions. On the other hand, the O_{3+4} , O_{3+4+8} and O_{st} arrangements exhibit greater genetic variability; they share some haplotypes, which, as expected, is an indication of the possibility of free genetic exchange since in these arrangement the *Od*h gene is not included in any inversion. Thus, some or all of these arrangements could be represented more than once in the colonizing sample and/or the *Od*h gene could exhibit a greater genetic exchange in them since it is located far away from any of their breakpoints. This latter possibility is supported by the data obtained in the Centralia population, which is the most recently collected sample (Solé et al. 2000) and in which the O_{3+4} , O_{3+4+8} and O_{st} arrangements are found at higher frequency. Both of these factors favor genetic exchange between these arrangements in this population.

Because the *Od*h gene does not overlap with any of the O_{3+4} , O_{3+4+8} or O_{st} arrangements, it is difficult to assign haplotypes to chromosomal arrangements in this case. They share five haplotypes and many different lethal and normal chromosomes carrying these arrangements could reach the Americas. The *Od*h gene sequence can be used to estimate the number of copies of a given inversion (or arrangement) if it is located inside or

very close to any of its breakpoints. However, it is not useful for those arrangements with breakpoints located far away from the *Od*h locus. Lethal genes behave in a similar way, showing associations with arrangements in many situations. It would be very useful to have other genetic markers, associated with the O_{3+4} and O_{3+4+8} arrangements available to estimate the number of these chromosomes that could be included in the genetic pool of the colonizers. In any case, the initial sample of chromosomes that reached America carried enough genetic variability to allow the establishment of *D. subobscura* and its expansion within both hemispheres.

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