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Taxonomic and molecular studies on *Drosophila sinobscura* and *D. hubeiensis*, two sibling species of the *D. obscura* group*

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Abstract

Allozyme electrophoresis and three different DNA sequences (*ATOC*180 satellite DNA, 5SrDNA repeats, and parts of the *Adh* gene) were used to compare the two closely related East Asian sibling species *Drosophila sinobscura* and *D. hubeiensis* producing fertile hybrids in the laboratory. The data were also applied to establish their phylogenetic relationships to the other species of the *D. obscura* group. Genetic divergence in 5SrDNA repeats and specifically in the *Adh* gene separate the two species clearly from each other and justify their species status. Both species are related to the European species of the *D. obscura* group but the presence of members of the *ATOC*180 satellite DNA family, specific and common to the species triad *D. ambigua*, *D. tristis* and *D. obscura*, in the genomic DNA of *D. sinobscura* and *D. hubeiensis* put the two sibling species in their close neighbourhood.

Key words: molecular systematics – species identification – sequence evolution – satellite DNA – 5S DNA – *alcohol dehydrogenase* – allozymes

Introduction

Taxonomists and systematists are frequently faced not only with the problem of discriminating between closely related, and thus morphologically almost indistinguishable, species but also of establishing their phylogenetic relationships. This is especially true for organisms with little morphological differentiation. The application of molecular techniques offers an opportunity to solve these issues. Actually, there is a growing body of papers that try to clarify debatable phylogenetic relationships deduced from morphological studies on the basis of molecular data. However, there exists a continuous and controversial discussion about the significance of such molecular approaches. A major and basic problem in this context is that 'gene trees' might frequently not reflect the true 'species trees'.

The evolution of no other animal genus has been studied as intensively as that of *Drosophila*. In the present paper the authors focus on two newly described species of the *Drosophila obscura* group, subgenus *Sophophora*: *D. sinobscura* (Watabe et al. 1996) and *D. hubeiensis* (Watabe and Sperlich 1997). According to morphological characters individuals from both species cannot be distinguished unambiguously from each other. Crosses between strains of the two species are successful in both directions producing fertile male and female offspring (Watabe and Sperlich 1997). They show morphological similarities to the palearctic species *D. subobscura*, *D. ambigua*, *D. tristis* and *D. obscura*. Sexual isolation to these species is low in laboratory experiments (Watabe et al. 1996). Although high morphological similarity and the apparent lack of isolation barriers under laboratory conditions indicate conspecificity, the karyotypes of the two species are different (Watabe and Sperlich 1997): *D. sinobscura* has 2n = 12 chromosomes (three pairs of small metacentric, two pairs of acrocentric and one pair of dot chromosomes). It resembles the karyotype of *D. obscura*. In contrast, *D. hubeiensis* has 2n = 10 chromosomes (three pairs of small metacentric, one pair of large metacentric and one pair of dot chromosomes), a karyotype that is also found in *D. ambigua* and *D. tristis*. The evolutionary origin of the difference between the karyotypes of *D. ambigua* and *D. tristis* on the one hand and that of *D. obscura* on the other hand can be either explained by the assumption of a fusion of two acrocentric chromosomes or a fission of one large metacentric chromosome (Felger and Pinsker 1987). The question remains open whether this difference indicates species status. In the present study, the authors try to answer this question with the help of molecular analyses applying allozyme variability and nucleotide sequence data. Parts of the *alcohol dehydrogenase* (*Adh*) gene, 5S rDNA repeats and highly repetitive satellite DNA (satDNA) were used for the sequence comparisons. The different markers were chosen for various reasons:

- The determination of allozyme variability is technically a rather simple and fast approach as the required methods are well established for *Drosophila* species. Given that numerous loci are studied, the data obtained can be taken as a reasonable overview of genome differentiation.
- Since Kreitman (1983) has sequenced the *Adh* gene from *D. melanogaster*, the nucleotide sequences of this protein coding gene were obtained and published for quite a number of *Drosophila* species; among them, several belong to the *D. obscura* group. The available data allowed polymerase chain reaction (PCR) primers to be designed (see Hagemann et al. 1996) for rapid amplification of parts of the *Adh* gene of *D. sinobscura* and *D. hubeiensis* and subsequent sequencing.
- Grau and Bachmann (1997) recently characterized the 5S rDNA repeats of 10 different species of the *D. obscura* group. Similar to the *Adh* genes, it appeared rather easy to 'complete' the data set with sequences from *D. sinobscura* and *D. hubeiensis*.
- Highly repetitive satellite DNA in the species of the *D. obscura* group was found to be either species specific (Bachmann et al. 1989; Gutknecht et al. 1995), specifically amplified in particular species (Bachmann et al. 1990) or restricted to closely related species only (Bachmann et al. 1992; Bachmann and Sperlich 1993). In *D. ambigua*, *D. tristis* and *D. obscura* the satellite DNA family *ATOC*180 proved to be a common

* Dedicated to Prof. Günther Osche on the occasion of his 70th birthday.

characteristic component of the genomes of these three species (Bachmann and Sperlich 1993). Thus, this sequence family should provide an excellent molecular tool to check if *D. sinobscura* and *D. hubeiensis* have to be considered as close relatives of this species triad.

Material and Methods

Drosophila strains

D. sinobscura: Collected in Chitou, Taiwan by H. Watabe, Sapporo, Japan.
D. hubeiensis: Collected near Hubei, China by H. Watabe, Sapporo, Japan.
D. subobscura H271: Collected near Helsinki, Finland by L. Serra, Barcelona, Spain.
D. guanche: Collected on Tenerife, Canary Islands, Spain by V. Cabrera, La Laguna, Spain.
D. madeirensis: Collected on Madeira Island, Spain by V. Cabrera, La Laguna, Spain.
D. obscura: Collected near Tübingen, Germany by W. Pinsker, Vienna, Austria.
D. tristis: Collected near Tübingen, Germany by W. Pinsker, Vienna, Austria.
D. ambigua: Collected near Vienna, Austria, by W. Pinsker, Vienna, Austria.
D. pseudoobscura: Laboratory strain from Department of Population Genetics, Tübingen, Germany.
D. miranda: Laboratory strain from Department of Medical Biology, Vienna, Austria.
D. persimilis: Laboratory strain from Department of Medical Biology, Vienna, Austria.
D. subsilvestris: Collected near Tübingen, Germany by D. Sperlich, Tübingen, Germany.
D. bifasciata Europe: Collected near Sunne, Sweden by D. Sperlich, Tübingen, Germany.
D. bifasciata Japan: Collected near Jyonzankei, Japan by H. Watabe, Sapporo, Japan.
D. imaii: Collected near Jyonzankei, Japan by H. Watabe, Sapporo, Japan.
D. tsukubaensis: Collected near Koganei, Japan by Takamori, Gakugei University, Tokyo, Japan.

Allozyme electrophoresis

Starch gel (SGE), cellulose acetate (CAE) and polyacrylamide electrophoreses (PAGE) were used. The SGE and enzyme staining techniques were those of Ayala et al. (1972). The CAE was performed according to the manual of Helena Laboratories, Ontario, Canada and PAGE was applied for amylase and esterase-6 as described by Davis (1964). The designation of allozymes is that of Lakovaara et al. (1976). The loci studied here are listed in Tables 1 and 2.

DNA isolation and cloning

Genomic DNA of *Drosophila* was isolated following the protocol of Preiss et al. (1988). Specific PCR primers were used for the amplification of the marker sequences.

*ATOC*180 *satDNA*: GATCCGATAAGAAACTG and AGGACATGCCACGCCTCCTA. The PCR products contain full length repeats of the *ATOC*180 satDNA family as described by Bachmann and Sperlich (1993).

5S rDNA repeats: CTTTAGATGGGGTACCGCTTGG and CCCCATCTAAGTACTAACCGC. The PCR products contain full length 5S DNA repeats as described by Grau and Bachmann (1997).

Adh gene: CTGGACTTCTGGGACAAGCG and TAGATGCCCGAGTCCCAGTG. The PCR products contain the 3′-section of exon 2, the complete intron 2, and the 5′-section of exon 3 as described by Hagemann et al. (1996).

The PCR followed standard procedures employing either the *Tfl*- (Epicenter) or *Taq* (Gibco-BRL) polymerase. The DNA fragments obtained were cloned into the pCRscript- (Stratagene) or pT7blue (Novagene) plasmid vector and transformed to *Escherichia coli* competent cells.

DNA sequencing

The AutoRead Kit (Pharmacia) was used for sequencing both strands according to the chain termination method (Sanger et al. 1977). Automatic sequencing was performed on an A.L.F. sequencer (Pharmacia).

Sequence analysis and computer programs

To analyze the primary sequence structure DNASIS 6.0 (Pharmacia) was used. The MEGA 1.0 program (Kumar et al. 1993) was employed for estimating the genetic distance values and for subsequent construction and plotting of the 'neighbor joining' (Saitou and Nei 1987) and 'maximum parsimony' (Eck and Dayhoff 1966) dendrograms.

Results

Allozymes

Only a few individuals from each species were used for allozyme analysis. Consequently, most enzyme loci studied were found to be monomorphic. The electrophoretic mobility of allozymes of 19 loci were determined and compared among the species. For all loci the relative migration index of the most frequent allozyme of *D. obscura* was set at 100 as a standard (Lakovaara et al. 1976). The results are given in Tables 1 and 2 separately for either SGE or CAE and PAGE. Genetic distance and identity matrices (Table 3) were calculated according to Nei (1972) and used for constructing an unrooted neighbour-joining dendrogram.

Five clades of related species can be detected in the resulting network: 1. *D. imaii* and *D. bifasciata* (two strains from Europe and Japan, respectively); 2. *D. sinobscura* and *D. hubeiensis*; 3. *D. subsilvestris*; 4. *D. ambigua*, *D. tristis* and *D. obscura*; and 5. *D. subobscura*, *D. tsukubaensis* and *D. pseudoobscura*. While the clustering of clades 1–4 might be reliable, clade 5 is certainly misinterpreted. Taking other phylogenetic analyses into account, clade 5 has to be split in three independent terminal branches, which is also indicated by the long branch lengths leading to *D. subobscura*, *D. tsukubaensis* and *D. pseudoobscura*, suggesting that the network of Fig. 1 should actually consist of seven clades. However, the phylogenetic relationships among these clades cannot be resolved in detail by the allozyme data obtained in this study.

(As the data are produced by different methods there are also different ways to treat them. However, the resulting dendrograms are basically identical. Table 3 and Fig. 1 are derived from results combining CAE and PAGE data.)

ATOC180 satDNA sequences

The *ATOC*180 satDNA family was described as being common to *D. ambigua*, *D. tristis* and *D. obscura* and absent in the genomes of all other *D. obscura* group species analyzed so far (Bachmann and Sperlich 1993). It appears remarkable that *ATOC*180 repeats could also be PCR-amplified from genomic DNA of *D. sinobscura* and *D. hubeiensis*. The sequence of seven independent repeats of *D. sinobscura* and two independent repeats of *D. hubeiensis* could be obtained. They have been deposited in 'GenBank' (accession numbers: U95540–U95548). These sequences were compared to the other *ATOC*180 sequences from *D. ambigua*, *D. tristis* and *D. obscura* (Table 4). The relationships resulting from this comparison are shown in the neighbor-joining dendrogram given in Fig. 2. Six of the

Table 1. Allozyme variability in 11 species of the *D. obscura* species group using starch gel electrophoresis. The numbers of allozymes observed refer to their relative mobility (*D. obscura* = 100) in the electric field

Species	sub	obs	tris	amb	susi	bifE	bifJ	ima	hub	sin	tsuk	pseu
Enzymes												
Acph	150	100	100	150	150	220	200, 220	200	100	100	100, 150	150
α-Gpdh	100	100	100	100	100	150	150	150	100	100	100	100
Aph 7	95	100	120	120	120	120	120	120	100	100	100	100
G-6-Pdh	100	100	100	80	?	110	110	110	110	110	80	80
6-Pgdh	100	100	100	100	100	100	100	100	100	100	100	100
Hk 1	100	100	100	100	140	140	140	140	140	140	20	140
Hk 3	200	200	200	200	210	200	200	195	195, 210	210	200	195
Idh	130	100	100	100	70	70	70	100	140	140	100	160
Lap	200	100	200	130	100	100	130	160	160	160	200	160
Mdh	95	100	160	210	160	210	210	210	110	110	60	110
Me	150	100	100	110	110	110	110	120	150	150	110	115
Pgm	100	100, 150	100	100	40	200	200	100	40	40	40	100
Pep 1	100	100	150	150	150	200	200	150	100	100	100	100
Pep 3	300	300	300	300	300	280	280	250	300	300	300	300
Phi	100	100	100	100	200	200	200	200	100	100	100	100
Tpi	100	100	100	100	100	130	130	130	100	100	100	100

sub, *D. subobscura*; obs, *D. obscura*; tris, *D. tristis*; amb, *D. ambigua*; susi, *D. subsilvestris*; bifE, *D. bifasciata* from Europe; bifJ, *D. bifasciata* from Japan; ima, *D. imaii*; hub, *D. hubeiensis*; sin, *D. sinobscura*; tsuk, *D. tsukubaensis*; pseud, *D. pseudoobscura*

Table 2. Allozyme variability in 11 species of the *D. obscura* species group using cellulose and polyacrylamide electrophoresis, respectively. The numbers of allozymes observed refer to their relative mobility (*D. obscura* = 100) in the electric field. For abbreviation of species names see Table 1

Species	sub	obs	tris	amb	susi	bifE	bifJ	ima	hub	sin	tsuk	pseu
Enzyme												
Acph	115	100	110	100	110, 115	130	130	115	100	100	110	110
Adh	60	100	100	100	80	80	80	80	80	80	60	60
α-Gpdh	100	100	100	100	100	150	150	150	100	100	100, 160	100
Aph 7	80	100	100	100	100	100	100	100	100	100	100	100
G-6-Pdh	100	100	100	100	100	100	100	100	100	100	110	100
6-Pgdh	100	100	100	90	100	100	100	100	100	100	100	100
Hk 1	100	100	100	100	100	100	100	100	100	100	100	80
Hk 3	100	100	100	100	100	100	100	100	100	100	100	100
Idh	110	100	100	100	90	90	90	100	110	110	100	120
Lap	200	100	200	160	100	100	100	160	160	160	160, 200	160
Mdh	95	100	160	210	160	210	210	210	110	110	60	105
Me	120	100	110	110	100	110	110	110	120	120	110	100
Pgm	100	100	100	100	40	200	180	100	40	40	40	100
Pep 1	100	100	120	120	120	200	220	180	100	100	100	100
Pep 3	50	100	100	100	100	30	30	30	100	100	50	100
Phi	100	100	100	100	100	120	120	120	100	120, 100	80	100
Tpi	100	100	100	100	100	120	120	120	100	100	100	100
Amy[a]	100	100, 120	70	100	60	10	10	10	10	10	70	100
Est 6[a]	120	100	100	90	80	80	90	100	100	80	110	110

[a] PAGE

seven sequences from *D. sinobscura* are very similar to those of *D. obscura* while one (*sin*180/5) appears almost like an 'outgroup' sequence. The sequences coming from *D. hubeiensis* form, on the other hand, a separate sequence cluster in the dendrogram. Also, one repeat of *D. sinobscura* and the two repeats *of D. hubeiensis* are significantly separated from the other six *ATOC*180 repeats of *D. sinobscura* and those of the three European species *D. ambigua*, *D. tristis* and *D. obscura*.

5S rDNA repeats

Three 5S DNA repeats were sequenced from *D. sinobscura* and four from *D. hubeiensis* (deposited in GenBank, accession numbers: U95801–U95807). Intraspecifically they show a high degree of sequence similarity typical for tandemly repeated DNA.

Consensus sequences derived for both species separately were aligned (Table 5) to those of *D. ambigua*, *D. tristis*, *D. obscura*, *D. subsilvestris*, *D. bifasciata*, and *D. imaii* published by Grau and Bachmann (1997). In the neighbor-joining dendrogram obtained (Fig. 3), four clades can be distinguished that are identical to those of the dendrogram derived from allozyme data: 1. *D. imaii* and *D. bifasciata*; 2. *D. subsilvestris*; 3. *D. sinobscura* and *D. hubeiensis*; and 4. *D. ambigua*, *D. tristis* and *D. obscura*. Again, the relationships between these four clades cannot be properly resolved by the 5S rDNA repeats. Numerous insertions/deletions have to be assumed to optimize the align-

Table 3. Genetic identities (above) and genetic distances (below diagonal) from CAE and PAGE (see Table 2). For abbreviations of species names see Table 1

	sub	obs	tris	amb	susi	bifE	bifJ	ima	hub	sin	tsuk	pseud
sub	—	0.500	0.474	0.474	0.395	0.211	0.211	0.312	0.526	0.500	0.421	0.526
obs	0.693	—	0.684	0.658	0.579	0.316	0.316	0.421	0.632	0.553	0.395	0.605
tris	0.747	0.379	—	0.684	0.605	0.316	0.316	0.474	0.526	0.447	0.526	0.526
amb	0.747	0.419	0.379	—	0.474	0.316	0.368	0.474	0.526	0.500	0.368	0.526
susi	0.930	0.547	0.502	0.747	—	0.474	0.421	0.342	0.579	0.605	0.368	0.500
bifE	1.558	1.153	1.153	1.153	0.747	—	0.842	0.684	0.368	0.447	0.263	0.211
bifJ	1.558	1.153	1.153	0.999	0.865	0.172	—	0.684	0.368	0.395	0.263	0.211
ima	1.153	0.865	0.747	0.747	1.073	0.379	0.379	—	0.474	0.447	0.342	0.316
hub	0.642	0.460	0.642	0.642	0.547	0.999	0.999	0.747	—	0.921	0.421	0.526
sin	0.693	0.593	0.804	0.693	0.502	0.804	0.930	0.804	0.082	—	0.421	0.500
tsuk	0.865	0.930	0.642	0.999	0.999	1.335	1.335	1.073	0.865	0.865	—	0.474
pseud	0.642	0.502	0.642	0.642	0.693	1.558	1.558	1.153	0.642	0.693	0.747	—

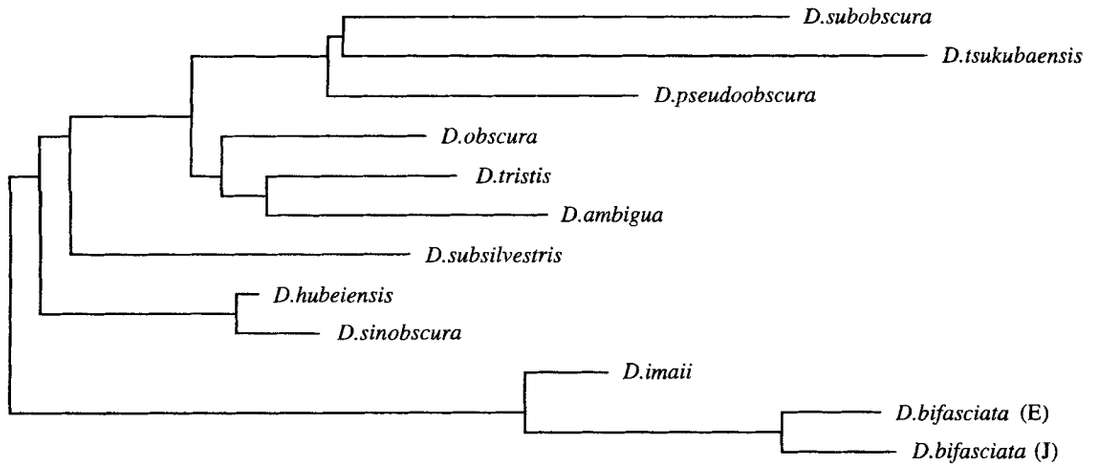

Fig. 1. Unrooted neighbor-joining dendrogram of *D. obscura* group species derived from allozyme data. Genetic distance values were calculated according to Nei (1972)

ment of the 5S rDNA repeats. Most of these gaps do not contribute to the topology of the dendrogram.

(The sequences of *D. subobscura*, *D. madeirensis*, *D. guanche* and *D. pseudoobscura* were not included as they already look highly diverged, and thus an alignment to the sequences used here appears to be at least partly random.)

Adh gene

The nucleotide sequences of an internal section of the *Adh* gene from *D. sinobscura* (430 bp) and *D. hubeiensis* (431 bp) were sequenced spanning parts of the exons 2 (124 bp) and 3 (246 bp) and the intron 2 (60 bp and 61 bp, respectively). Furthermore, the homologous fragments of the *Adh* gene of *D. obscura* (453 bp) and *D. tristis* (428 bp) were sequenced as they were not available in the nucleotide sequence database. (The sequences have been deposited in GenBank, accession numbers: U90953–U90956.) Together with the respective *Adh* regions of *D. melanogaster* (Kreitman 1983), *D. ambigua* (Marfany and Gonzalez-Duarte 1991), *D. subobscura* (Marfany and Gonzalez Duarte 1992), *D. madeirensis* and *D. guanche* (Marfany and Gonzalez Duarte 1993), *D. bifasciata* and *D. imaii* (Hagemann et al. 1996), *D. pseudoobscura* (Schaeffer and Aquadro 1987), *D. persimilis* and *D. miranda* (Schaeffer and Miller 1991) they were used (see Table 6) for the construction of a neighbor-joining dendrogram (Fig. 4). Five clades, each consisting of two or three closely related species, can be discriminated similar to those described in the allozyme and the 5S DNA studies. They are: 1. *D. imaii* and *D. bifasciata*; 2. *D. pseudoobscura*, *D. persimilis* and *D. miranda*; 3. *D. subobscura*, *D. madeirensis* and *D. guanche*; 4. *D. sinobscura* and *D. hubeiensis*; and 5. *D. ambigua*, *D. tristis* and *D. obscura*. *Drosophila melanogaster* serves as an outgroup. Again, the relationships of these five clades remain uncertain indicated by bootstrap values of less than 50. The topology of the tree does not change significantly when either the intron or substitutions at the third codon position are excluded from the analysis.

Discussion

In order to clarify the species status of the two recently described strains *D. sinobscura* and *D. hubeiensis*, as well as their phylogenetic position in the dendrogram of the *D. obscura* group, four different molecular data sets were used: allozyme variability and three sets of nucleotide sequence data (an *Adh* gene section, 5S rDNA repeats, and the highly repetitive *ATOC*180 satellite DNA). Dendrograms were generated from all four data sets and compared for congruence and significance.

On the basis of morphological characters *D. sinobscura* and *D. hubeiensis* can be grouped close to *D. subobscura* and the species triad *D. ambigua*, *D. tristis*, and *D. obscura*. No reliable

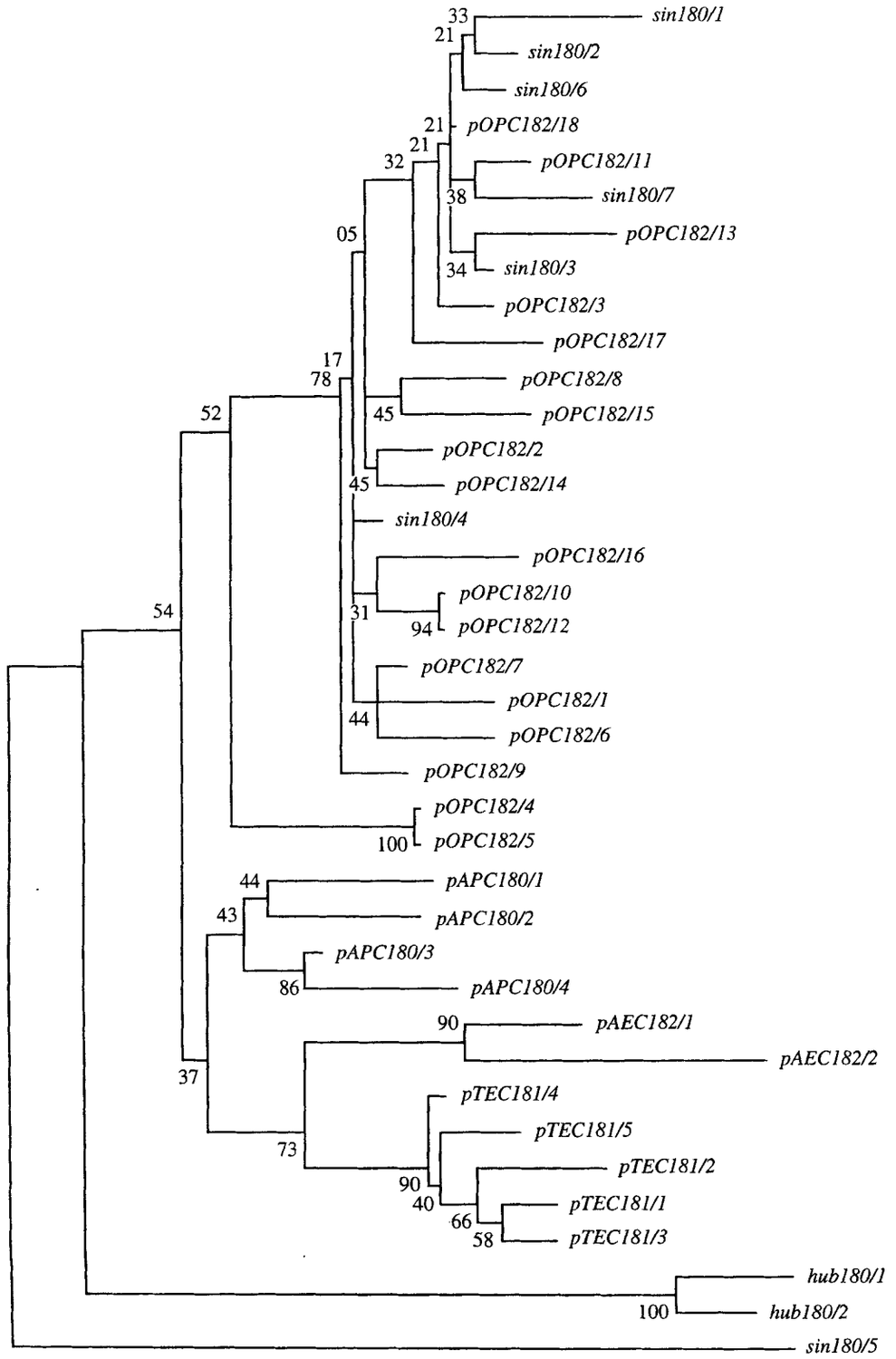

Fig. 2. Unrooted neighbor-joining dendrogram of *ATOC*180 satellite DNA sequences. Genetic distance values were calculated according to Kimura (1980). The number at each node represents the bootstrap value per 100 replications

morphological differences could be found to distinguish these two species (Watabe and Sperlich 1997).

The *ATOC*180 satDNA data strongly support a close relationship of *D. sinobscura* and *D. hubeiensis* to the species triad *D. ambigua*, *D. tristis* and *D. obscura*. So far, *ATOC*180 sequences were only detected in *D. ambigua*, *D. tristis* and *D. obscura* (Bachmann and Sperlich 1993). The presence of this sequence family in *D. sinobscura* and *D. hubeiensis* indicates common ancestry as there are no examples of satDNA families occurring in paraphyletic species. Furthermore, the *ATOC*180 consensus sequences of *D. sinobscura* and *D. obscura* (both species with 2n = 12 chromosomes) are practically identical.

Table 4. Nucleotide sequences of *ATOC*180 satellite DNA repeats. * indicate gaps introduced to optimize the alignment, # indicate PCR primer sequence. *pOPC*182/1-18 (*D. obscura*), *pAPC*180/1-4 and *pAEC*180/1-2 (*D. ambigua*), and *pTEC*181/1-5 (*D. tristis*) are taken from Bachmann and Sperlich (1993)

	10	20	30	40	50	60	70	80	90	100
*pOPC*182/1	CTGCAGAGTT	AGAGTGAAAA	AGGGATATTT	ACTGGCAG*AT	TACGT*TATA	GTAGCTATAT	CCTTTATTTC	CTGCAATCCA	AATTGATTGC	AAAAGAT*CC
*pOPC*182/2	----------	---------C	-----G--A-	-----A--*--	-----G----	--GA------	-------G--	---A------	----------	--G-C--*--
*pOPC*182/3	----------	---------C	-A--------	------C-*GA	-----*----	--G-------	-A-----G--	----------	----------	-------*--
*pOPC*182/4	----------	-----A---C	----------	-A-T----*T	-----*--C-	TGG-------	-------G--	---T------	--------A-	---CC--*AA
*pOPC*182/5	----------	-----A---C	----------	-A-T----*T-	-----*--C-	TGG-------	-------G--	---T------	--------A-	---CC--*AA
*pOPC*182/6	----------	----------	----------	-------*--	-----*----	--G-------	-------G--	----C-----	----------	-------*--
*pOPC*182/7	----------	----------	G---------	-------*--	-----*----	--G-------	-------G--	---A------	----------	-------*--
*pOPC*182/8	----------	---------C	---------A	-------*--	---A-*----	--G-------	-------G--	----C-----	---G---A--	-------*--
*pOPC*182/9	----------	---------C	----------	-------*--	-----*----	--G-------	-------G--	----------	--------T-	-------*--
*pOPC*182/10	----------	---------C	----------	-------*--	----C*A---	--G-------	-------G--	----C-----	----------	-------*--
*pOPC*182/11	----------	--------GC	----------	------C-*GA	-----*----	--G-------	-------A--	----C-----	----------	-------*--
*pOPC*182/12	----------	---------C	----------	--------*--	----C*A---	--G-------	-------G--	----C-----	----------	-------*--
*pOPC*182/13	----------	G-------GT	----------	------C-*GA	-----*----	--G--A----	-------G--	----C-----	----------	-------*--
*pOPC*182/14	----------	--------TC	T---------	--A--A-A*--	-----*----	--G--A----	-------G--	-------T--	----------	-------*--
*pOPC*182/15	----------	---------C	----------	-------*--	-----*----	--G-------	-------G--	----------	----------	-------*--
*pOPC*182/16	----------	---------C	----------	-----T--*--	-----*A---	--G--A----	-------G--	----C---T-	----------	-------*A-
*pOPC*182/17	----------	--------GC	----------	------C-*G-	-----*----	--G-------	--A----G--	----C-----	---------G	-------*--
*pOPC*182/18	----------	--------GC	----------	------C-*GA	-----*----	--G-------	-------G--	----------	----------	-------*--
*pAPC*180/1	----------	-T-------C	------*---	---T*--T*T-	-----*---T	A-GT------	---C---G-G	---T-----G	-T-A------	---C---*--
*pAPC*180/2	--------G-	-T-------C	------*---	---T*--T*T-	-----*----	A-G-------	---C--CG--	---T------	---G------	---C---*--
*pAPC*180/3	----------	-T-------C	------*---	---T*--T*T--	-----*----	--G-------	---C------	---T--AA--	---G------	---C---*--
*pAPC*180/4	----------	-T-------C	------*A--	---T*--T*TG	-----*----	A-G-------	---C---G--	---T-----G	---A------	---C---*--
*pAEC*180/1	---TTT-AA-	-TG---T--C	------*--A	-T-TG-G*T-	-----*A---	--G-------	-A-----G--	---T------	-------C--	---C---*-A
*pAEC*180/2	---TT--AA-	-TGA--T--C	------*--C	---T-G-T*T-	-----*----	--G-------	A--C-TAGG-	-		
*pTEC*181/1	---TT-----	-TG------C	-----G*---	-*-T--G-*T-	A----*----	-AG-------	-----C-G--	---A------	----------	---C---*--
*pTEC*181/2	---TT-----	-TG------C	-----G*---	---T--G-*T-	-----*----	-AG-------	--C----G--	T--A------	----------	-C-C---*--
*pTEC*181/3	--ATT-----	-TG------C	-----G*---	---T--G-*T-	----C*----	-AG-------	----*--G--	---A------	------CG--	-------*--
*pTEC*181/4	---TT-----	-TG------C	-----G*---	---T--G-*G-	-----*----	--G-------	-------G--	---A------	----------	---G---*--
*pTEC*181/5	A--TT-----	-TT------C	-----G*---	---T-GG-*T-	-----*----	--G-------	-T-----G--	---A------	----------	---C---*--
*hub*180/1	###-----G-	-T--A---TC	-A---C*---	G--T---ATT-	-CGT-*ATA-	*CG--G----	A------G--	---GG-----	-TG-C-----	---C---*--
*hub*180/2	###-T---G-	-T--A----C	-A---C*---	G--T---ATT-	-CGT-*-TA-	*CG-------	-------G-T	---GG-----	-TG-C-----	---C---*--
*sin*180/1	###---TT--	--------GC	*-----T---	-G----C-*GA	-----*----	--G-------	----------	----------	----------	-------*--
*sin*180/2	###-------	--------GC	----------	---A--C-*GA	-----*----	--G-------	-------G--	----------	----------	-------*T-
*sin*180/3	###-------	--------GC	----------	------C-*GA	-----*----	--G-------	-------G--	----C-----	----------	-------T--
*sin*180/4	###-------	T-------C	----------	-------*--	-----*----	--G-------	-------G--	----------	----------	-------*--
*sin*180/5	###--A----	-TG-------	----G-T--*	---T-A-A*T-	A----*----	A-T--AG---	AGC-C--C-A	GT-G-G-AA-	--AAA-A-T-	C---C-AT--
*sin*180/6	###---T---	--------GC	---------A	------C-*GA	-----*----	--G-------	-------G--	----------	----------	-------*--
*sin*180/7	###-------	--------GC	----------	-----C-*GA	-----*----	--G-------	A------A--	----C-----	----------	-------*--

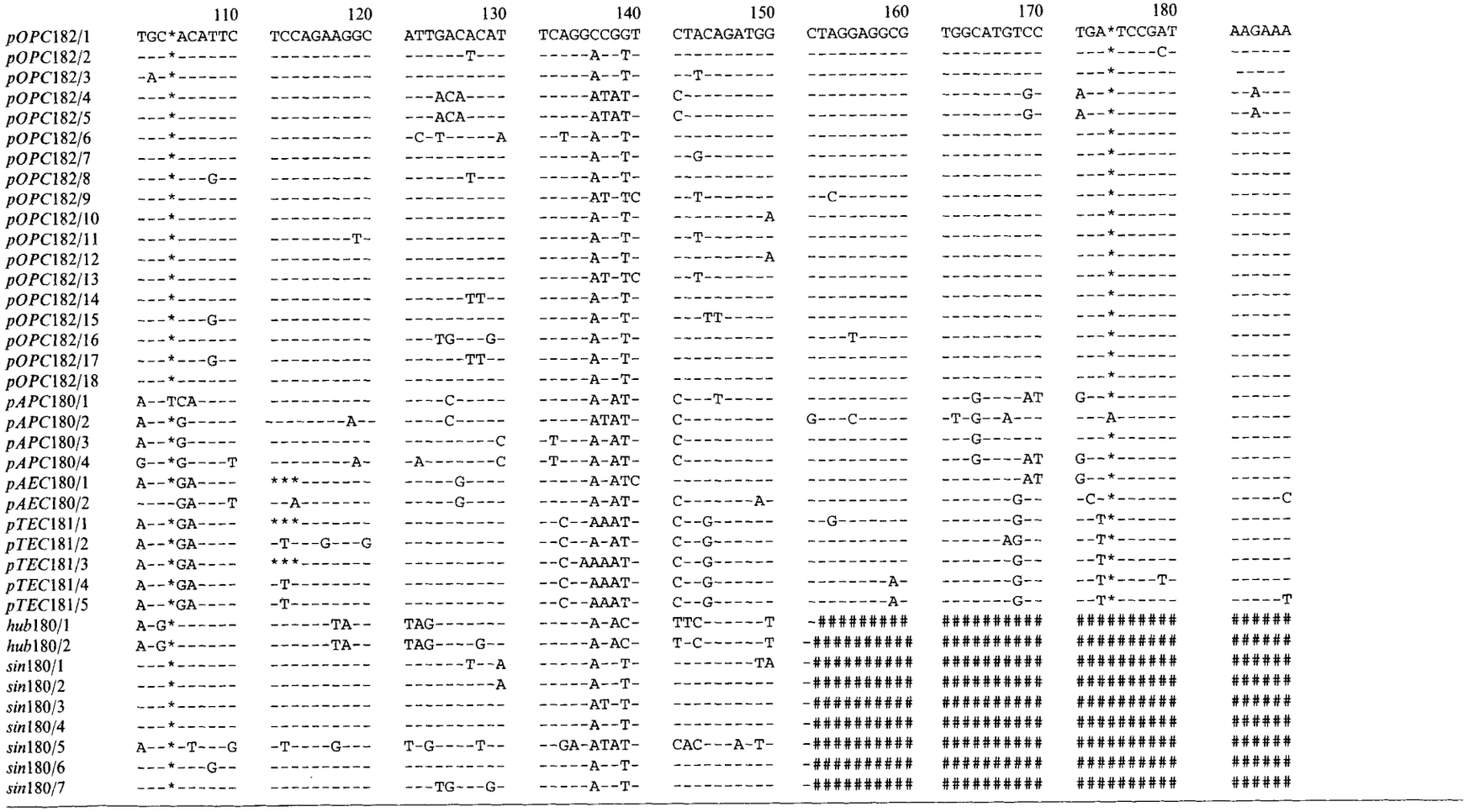
110 120 130 140 150 160 170 180
pOPC182/1 TGC*ACATTC TCCAGAAGGC ATTGACACAT TCAGGCCGGT CTACAGATGG CTAGGAGGCG TGGCATGTCC TGA*TCCGAT AAGAAA
pOPC182/2 ---*------ ---------- ------T--- -----A--T- ---------- ---------- ---------- ---*----C- ------
pOPC182/3 -A-*------ ---------- ---------- -----A--T- --T------- ---------- ---------- ---*------ -----
pOPC182/4 ---*------ ---------- ---ACA---- -----ATAT- C--------- ---------- --------G- A--*------ --A---
pOPC182/5 ---*------ ---------- ---ACA---- -----ATAT- C--------- ---------- --------G- A--*------ --A---
pOPC182/6 ---*------ ---------- -C-T-----A --T--A--T- ---------- ---------- ---------- ---*------ ------
pOPC182/7 ---*------ ---------- ---------- -----A--T- --G------- ---------- ---------- ---*------ ------
pOPC182/8 ---*---G-- ---------- ------T--- -----A--T- ---------- ---------- ---------- ---*------ ------
pOPC182/9 ---*------ ---------- ---------- -----AT-TC --T------- --C------- ---------- ---*------ ------
pOPC182/10 ---*------ ---------- ---------- -----A--T- ---------A ---------- ---------- ---*------ ------
pOPC182/11 ---*------ --------T- ---------- -----A--T- --T------- ---------- ---------- ---*------ ------
pOPC182/12 ---*------ ---------- ---------- -----A--T- ---------A ---------- ---------- ---*------ ------
pOPC182/13 ---*------ ---------- ---------- -----AT-TC --T------- ---------- ---------- ---*------ ------
pOPC182/14 ---*------ ---------- ------TT-- -----A--T- ---------- ---------- ---------- ---*------ ------
pOPC182/15 ---*---G-- ---------- ---------- -----A--T- ---TT----- ---------- ---------- ---*------ ------
pOPC182/16 ---*------ ---------- ---TG---G- -----A--T- ---------- ----T----- ---------- ---*------ ------
pOPC182/17 ---*---G-- ---------- ------TT-- -----A--T- ---------- ---------- ---------- ---*------ ------
pOPC182/18 ---*------ ---------- ---------- -----A--T- ---------- ---------- ---------- ---*------ ------
pAPC180/1 A--TCA---- ---------- ----C----- -----A-AT- C---T----- ---------- ---G----AT G--*------ ------
pAPC180/2 A--*G----- ---------A-- ----C----- -----ATAT- C--------- G---C----- -T-G--A--- ---A------ ------
pAPC180/3 A--*G----- ---------- ---------C -T---A-AT- C--------- ---------- ---G------ ---*------ ------
pAPC180/4 G--*G----T --------A- -A-------C -T---A-AT- C--------- ---------- ---G----AT G--*------ ------
pAEC180/1 A--*GA---- ***------- -----G---- -----A-ATC ---------- ---------- --------AT G--*------ ------
pAEC180/2 ----GA---T --A------- -----G---- -----A-AT- C-------A- ---------- -------G-- -C-*------ -----C
pTEC181/1 A--*GA---- ***------- ---------- --C--AAAT- C--G------ --G------- -------G-- --T*------ ------
pTEC181/2 A--*GA---- -T---G---G ---------- --C--A-AT- C--G------ ---------- ------AG-- --T*------ ------
pTEC181/3 A--*GA---- ***------- ---------- --C-AAAAT- C--G------ ---------- -------G-- --T*------ ------
pTEC181/4 A--*GA---- -T-------- ---------- --C--AAAT- C--G------ --------A- -------G-- --T*----T- ------
pTEC181/5 A--*GA---- -T-------- ---------- --C--AAAT- C--G------ --------A- -------G-- --T*------ -----T
hub180/1 A-G*------ ------TA-- TAG------- -----A-AC- TTC------T -########## ########## ########## ######
hub180/2 A-G*------ ------TA-- TAG----G-- -----A-AC- T-C------T -########## ########## ########## ######
sin180/1 ---*------ ---------- ------T--A -----A--T- --------TA -########## ########## ########## ######
sin180/2 ---*------ ---------- ---------A -----A--T- ---------- -########## ########## ########## ######
sin180/3 ---*------ ---------- ---------- -----AT-T- ---------- -########## ########## ########## ######
sin180/4 ---*------ ---------- ---------- -----A--T- ---------- -########## ########## ########## ######
sin180/5 A--*-T---G -T----G--- T-G----T-- --GA-ATAT- CAC---A-T- -########## ########## ########## ######
sin180/6 ---*---G-- ---------- ---------- -----A--T- ---------- -########## ########## ########## ######
sin180/7 ---*------ ---------- ---TG---G- -----A--T- ---------- -########## ########## ########## ######

Table 5. Consensus sequences of 5S rDNA repeats from *D. sinobscura* and *D. hubeiensis*. * indicate gaps introduced to optimize the alignment, # indicate PCR primer sequence. Other sequences taken from Grau and Bachmann (1997)

	10	20	30	40	50	60	70	80	90	100
D. ambigua	GCTTGGGAAC	ACCGCGTGTT	GTTGGCTTCG	TCACCAAC**	TTTTTGCCTT	TTACCGCTTT	TTCG******	TTTCGTTTCG	TTTTTA****	AT*TTTTTTT
D. obscura	######----	----------	----------	--------**	----------	---------*	******----	----------	------**AT	T-*-------
D. tristis	######----	----------	----------	--------TT	----------	-----A---*	******----	--CTA-----	------AACT	--T-------
D. hubeiensis	######----	----------	----------	--------**	----------	---------*	******----	--*-------	-----C**AT	T-T--A----
D. sinobscura	######----	----------	----------	--------**	----------	---------*	******----	--*-------	-----C**AT	T-T--A----
D. bifasciata	######----	----------	----------	--------**	----------	--T****---C-	--A*CCCCTT	---------T	-----***CT	T-T--AACC-
D. imaii	######----	----------	----------	--------**	----------	--T****---C-	--A*CCCCTT	---------T	-----***CT	T-T--AACC-
D. subsilvestris	######----	----------	------C---	--------**	----------	--T-------	---*CTTTAG	----------	-----AA*C*	--T-AA----

	110	120	130	140	150	160	170	180	190	200
D. ambigua	TCA*TTTTCC	AGTTTTAGTT	*GTCTTTGGG	CTTATTTTGG	CAAAG****G	TACATGGACA	AAATGAATAT	TAAATGAAAA	TTAAAGCTAA	AGGTA*ATCA
D. obscura	---*-*****	**--------	*-C-C--A--	----------	-----****C	------C-A-	----T-----	----------	------T-**	****T*-C--
D. tristis	---*----*-	-T--------	G-C-------	----------	-----****-	-T-----GA-	----T-----	----------	--G-------	-----*----
D. hubeiensis	---*----T-	GT-------C	*-C--C-A--	----------	-----****-	----------	----T-----	--GG------	-----A-***	**T--*----
D. sinobscura	-G-*----T-	GT-------C	*-C--C-A--	----------	-----GTAT-	----------	----T-----	--GG------	-----A-***	**TA-*----
D. bifasciata	--TA----T-	*A-A--***-	CAC----AA-	T---------	-----****-	----------	----T-----	--TC------	-----AA---	-T--TC----
D. imaii	--TA----T-	*A-A--***-	CAC----AA-	T---------	-----****-	----------	---CT-----	--TC------	-----AT---	-T--TC----
D. subsilvestris	---*----T-	GT--------	*-C--C-A--	--------T-	A---T****-	C-------A-	----T-G---	---T-C---*	--------**	****T*----

	210	220	230	240	250	260	270	280	290	300
D. ambigua	AAACATAATG	TTTGCTGGCG	AACGGCATTT	GG********	******TTTT	TCTTTTCAAT	AAAGTAATA*	*AATTAAGGG	CATGATGG**	**********
D. obscura	--CTG-----	-----AC-T-	----------	--********	******----	--------G-	---------*	*------A--	--G---A-CC	AAACGGGCAT
D. tristis	---CG-----	----------	--T-------	--********	******----	--------G-	---------*	*---C-----	------A-**	**********
D. hubeiensis	-T-TG---A-	-----AC-TA	-----A--G-	--********	******-G-G	GT-----T*-	TTCAAT---*	*----C-T--	T-G---A-CC	TTACTGGCAT
D. sinobscura	-T-TG---A-	-----AC-TA	-----A--G-	--********	******-G-G	GT-----T*-	CTCAAT---*	*----C-C--	T-G---A-CC	TTACTAACAT
D. bifasciata	---TG-****	*********-	----------	--TTTTCAAT	TTTCCT----	C---------	-******--*	*---ACCT--	--G*******	***CGG****
D. imaii	----T-****	*********-	----------	--TTCTCCTT	TTTCCA----	C---------	-******-T*	*---AC-T--	T-G*******	***CAG----
D. subsilvestris	---TG-AA-T	-----GC-T-	----------	T-********	******----	G********-	-TGT-----T	G-G--T-T-A	--G--CT-CC	ATACAGGCAT

	310	320	330	340	350	360	370	380	390	400
D. ambigua	CCATACAATC	CTTGCCAGTA	GTTGCAAGTC	ATTTGTAAAT	GTATCGGTTT	CGTTGAACCG	TTTGGACTAA	AAATAGTCTT	GTTAGTTTTG	GAAAACCAAC
D. obscura	----------	---A-****-	-A--------	---C------	--C-------	----------	----------	----------	A--G---A--	----------
D. tristis	--T-------	------T---	-A--------	---C-----A	--C-T-----	----------	-----G----	----------	C--G---AC-	--------G-
D. hubeiensis	----------	---A------	-A-TTTT---	T*****---A	**********	**********	*A-A----C-	--T-------	---C------	----------
D. sinobscura	----------	---A------	-A-TTTT---	T*****---A	**********	**********	*G-A----C-	--T-------	---C------	---------G
D. bifasciata	T------*--	--CC-ATCGC	-G---*****	**-*------	----------	**********	******----	-T--------	--A----C--	----------
D. imaii	T------*--	--CC-ATCGC	-G---*****	**-*------	----------	**********	******----	-T--------	--A--C-C--	----------
D. subsilvestris	----------	----------	----TGG---	-C-----GG-	----------	--------TT	A------C--	-T--------	----------	-*--------

	410	420	430	440	450	460	470	480	490	500
D. ambigua	GTTCAGCTCT	CAGGCTTTCG	CCAACGACCA	TACTACGCTG	AATACATCGG	CTCTCGTCCG	ATCACCGAAA	TTAAGCAGCG	TCGGGCGCGG	TTAGTAACTT
D. obscura	----------	----------	----------	----------	----------	T---------	----------	----------	------####	##########
D. tristis	---T------	----------	----------	----------	----------	T---------	----------	----------	------####	##########
D. hubeiensis	----------	----------	----------	----------	----------	T---------	----------	----------	------####	##########
D. sinobscura	----------	----------	----------	----------	----------	T---------	----------	----------	------####	##########
D. bifasciata	---T---C--	*-A-------	----------	----------	----------	----------	----------	----------	------####	##########
D. imaii	---T---C--	*TA-------	----------	----------	----------	----------	----------	----------	------####	##########
D. subsilvestris	---T------	*-A-------	----------	----------	----------	----------	----------	----------	------####	##########

	510	
D. ambigua	AGATGGGGTA	CC
D. obscura	##########	##
D. tristis	##########	##
D. hubeiensis	##########	##
D. sinobscura	##########	##
D. bifasciata	##########	##
D. imaii	##########	##
D. subsilvestris	##########	##

Table 6. Nucleotide sequences of segments of the *Adh* gene. * indicate gaps introduced to optimize the alignment, | indicate exon–intron borders. For references of sequences taken from other authors see text

```
                          10         20         30         40         50         60         70         80         90        100
D. pseudoobscura  CAAGGGCGGC CCAGGCGGCA TCATTTGCAA CATTGGCTCC GTCACCGGTT TCAATGCCAT CTACCAGGTG CCCGTTTACT CCGGCTCCAA GGCGGCGGTG
D. miranda        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----A---- A---------
D. persimilis     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
D. imaii          ---------- -----T---- ---------- ---------- ---------- ---------- ---------- -----C---- -T---A---- ------T--T
D. bifasciata     ---------- -----T---- ---------- ---------- ---------- ---------- ---------- -----C---- -T---A---- ------T---
D. ambigua        ---------- ---------- ---------- ---------- ---------- ---------- ---------- -----C---- -T---AG--- ---C--C---
D. tristis        ---C------ ---------- ---------- ---------- --------C- ---------- ---------- ---------- -T---AG--- ---C--C---
D. obscura        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -T---AG--- ---C--C--C
D. sinobscura     ---------- ---------- ---------- ---------- ---------- ---------- -----G---- -----C---- -T---A---- ---T--T---
D. hubeiensis     ---------T ---------- ---------- ---------- ---------- -----A---- ---------- ---------- -T---A---- ---T--T---
D. guanche        ---------- -----T---- ---------- ---------- --T------- -T-------- ---------- ---------- -T---AG--- ----------
D. madeirensis    ---------- -----T---- ---------- ---------- --T------- -T-------- ---------- --T------- -T---AG--- ----------
D. subobscura     ---------- -----T---- ---------- ---------- --T------- -T-------- ---------- ---------- -T---AG--- ----------
D. melanogaster   ---------T --C--T--T- ----C----- ------A--- -----T--A- ---------- ---------- -----C---- -----A---- ---C--C---

                          110        120        130        140        150        160        170        180        190        200
D. pseudoobscura  GTTAACTTTA CCTCCTCCCT GGCG|GTAAGA A*ATCC**** TACAACTA*T CCTA*****C TCCCCAAAAC TAAATATTTC TTT******* *********G
D. miranda        ---------- ---------- ----|-----T -*----**** A--GTT-T*- T---******- ----T*---- -G-------- C--******* *********C
D. persimilis     ---------- ---------- ----|------ -*----**** --------*- --C-***AAA ---------- ---------- -AA******* *********A
D. imaii          --------C- ---G------ ----|------ -A-CTT**** A-GCCA-TC- T-C-****** **G-TG---- ---****--- -C-******* *****GCTCT
D. bifasciata     --------C- ---G---T-- ----|------ -A-CTT**** A-ACCA-TC- T-C-****** **G-TG--T- ---****--- -C-******* *****GCTCT
D. ambigua        --G-----C- --AG------ ----|------ -A--TG**** ATT-GA--A- T-C-TCAGA- *AT-AG---- ---****--- ---TTTTT** *****GTT**
D. tristis        --G-----C- --AG------ ----|------ -*---T**** ATT-CA--A- T-C-TCAGC- *AA-AG---- ---*****--T ---******* *****GTT**
D. obscura        --G-----C- --AG------ ----|------ -A--ATATTT ATT-CA--A- T-C-TCAGC- AAA-T----G ---****--- ---TGTTTTT TTGTTGTTGC
D. sinobscura     --------C- ---G------ ----|------ -A-*AT***C A*--CA-**- T---TG**C- *-A-TG---- ---****--- C--******* *****GTGCC
D. hubeiensis     --------C- ---G------ ----|-----C -A-CAG***C A*T-CA-**- TT--TG**C- *-A-TG---- ---****--T ---******* *****GTGCC
D. guanche        --A-----C- --AG------ ----|-----C -C---T***C ATA-G*-**- T---TT**** *-T-TG---- ---****--- --AACTTA** *******TCC
D. madeirensis    --------C- --AG------ ----|-----C GC---TCT*C AT--CA-CTC T---TT**** *-G-AG---- ---****--- --AACTTA** *******TC-
D. subobscura     --------C- --AG------ ----|-----C GC---TCT*C AT--CA-CTC T---TT**** *-G-AG---- ---****--- --AACTTA** *******TCC
D. melanogaster   --C-----C- --AG------ ----|-----T TG---AAAGG A-ACG-A-AG TT-TCAAGAA AAAA------ --A****--T G**ATTTAT* **********

                          210        220        230        240        250        260        270        280        290        300
D. pseudoobscura  AAATCCCATA G|AAACTGGCT CCCATTACTG GTGTCACTGC TTACACTGTC AATCCTGGCA TCACTAAGAC CACTCTGGTC CACAAATTCA ACTCGTGGCT
D. miranda        -----T---- -|--------- --T------- ---------- ---------- ---------- ----C-G--- ---------- ---------- ----------
D. persimilis     T-T------- -|--------- ---------- ---------- ---------- ---------- ----C-G--- ---------- ---------- ----------
D. imaii          ------T--- -|-----T--A ---------- -------C-- ---------G ---------- ----C-G--- ---------- ---------- ----------
D. bifasciata     ---A--T--- -|-----T--A -----C---- -------C-- ---------G ---------- ----C-G--- ---------- ---------- ----------
D. ambigua        **T-T-TT-- -|-----T--A -----C---- ----G--C-- C--------- ---------- -T--C----- ---G------ -----G---- ----------
D. tristis        *****-TT-- -|-----T--A -----C--C- ----G--C-- ---------- ---------- -T--C----- ---G------ ---------- ----------
D. obscura        ------TT-- -|-----T--A --A--C---- ----G--C-- ---------- ---------- -T--C----- ---G------ ---------- ----------
D. sinobscura     ------TT-- -|-----T--A -----C--C- -------C-- A--------G ---------- ----C-G--- ---------- ---------- ----------
D. hubeiensis     ------TT-- -|-----A--A -----C---- -------C-- C--------G ---------- ----C-G--- ---A------ ---------- ----------
D. guanche        -----TTT-- -|-----T--A -----C---- -A-----C-- A--------G -----G---- ----C----- ---------G ---------- ----------
D. madeirensis    -----GTT-- -|-----T--A -----C---- -A-----C-- A--------G -----G---- ----C----- ---------G ---------- ----------
D. subobscura     -----GTT-- -|-----T--A -----C---- -A-----C-- A--------G -----G---- ----C----- ---------G ---------- ----------
D. melanogaster   --CA--TT-- -|--------C ---------C- -C--T--C-- ------C--G --C--C---- ----CCGC-- ---C-----G -----G---- ----C---T-
```

```
                          310        320        330        340        350        360        370        380        390        400
D. pseudoobscura  GGATGTGGAG CCCCGTGTGG CGGAGAAGCT GCTCGAGCAT CCCACCCAGA CCTCTCAGCA GTGCGCCGAG AACTTTGTGA AGGCCATTGA GCTGAACAAG
D. miranda        ---------- ----T----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
D. persimilis     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -------C-- ----------
D. imaii          ---------- ---A-G--T- ---------- -T-G--A--- ---------- -G--G-T--- ---T------ --------C- ---------- ----------
D. bifasciata     ---------- ---A-G--T- ---------- -T-G--A--- ---------- ----G-T--- ---------- --------C- ---------- ----------
D. ambigua        ---------- --GAA----- -C-------- ---G------ ---------- ---------- ---T------ --------C- ---------- ----------
D. tristis        ---------- ---AA----- -C-------- ---G-----C ---------- ---------- ---T------ --------C- ---------- ----------
D. obscura        ---------- ---AAG--A- -C-------- ---G------ ---------- -A-------- ---T------ ---------- ---------- ----------
D. sinobscura     ---------- ---AAG---- ---------- ---------- ---------- ---------- ---T------ --------C- ---------- ----------
D. hubeiensis     ---------- ---AAG---- ---------- ---------- ---------- ---------- ---T------ --------C- ---------- ----------
D. guanche        ---------- ---A-A---- ---------- -T-G------ ---------- ---------- ---T------ --------C- ---------- ----------
D. madeirensis    ---------- ---A-A---- ---------- ---G------ ---------- ---------- ---T------ --------C- ---------- ----------
D. subobscura     ---------- ---A-A---- ---------- ---G------ ---------- ---------- ---T------ --------C- ---------- ----------
D. melanogaster   ------T--- ----AG--T- -T-------- C--G-CT--- ---------C -A--GTT-GC C--------- -----C--C- ----T--C-- A------C--

                          410        420        430        440        450
D. pseudoobscura  AACGGTGCCA TCTGGAAGTT GGATCTGGGC ACCTTGGAGC CCATCACATG GACCCAG
D. miranda        ---------- ---------- ----T----- ---------- ---------- -------
D. persimilis     ---------- ---------- ----T----- ---------- ---------- -------
D. imaii          --T--A---- --------C- ----T----- --TC------ ---------- -------
D. bifasciata     --T--A---- --------C- ----T----- --TC------ ---------- -------
D. ambigua        -----C---- -------TC- ---CT----- --TC------ ---------- -------
D. tristis        ---------- --------C- ----T----- --TC------ ---------- -------
D. obscura        --T------- --------C- ----T----- --AC------ -G-------- -------
D. sinobscura     --T------- --------C- ----T----- --TC------ ---------- -------
D. hubeiensis     --T------- --------C- ----T----- --TC------ ---------- -------
D. guanche        --T-----T- -------AT- ---CT----A --TC------ ---------- ----A--
D. madeirensis    --T------- -------AT- ---CT----A --TC-----T ---------- ----A--
D. subobscura     --T------- -------AT- ---CT----A --TC-----T ---------- ----A--
D. melanogaster   -----A---- -------AC- ---CT----- ---C-----G -----CAG-- ----A--
```

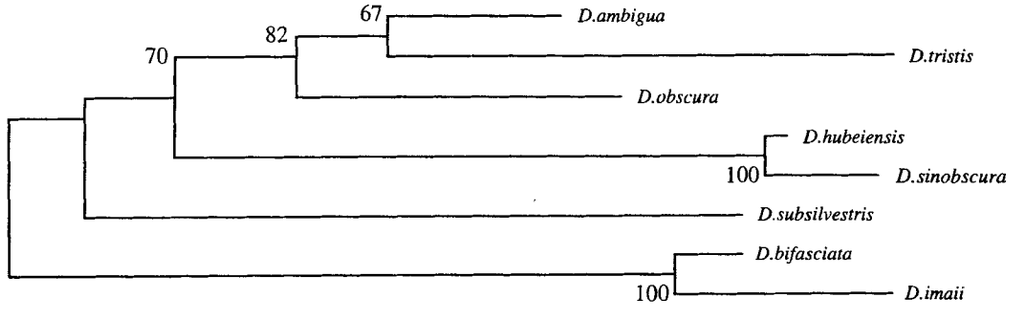

Fig. 3. Unrooted neighbor-joining dendrogram of the consensus sequences of 5SrDNA repeats of several *D. obscura* group species. Genetic distance values were calculated according to Kimura (1980). The number at each node represents the bootstrap value per 100 replications

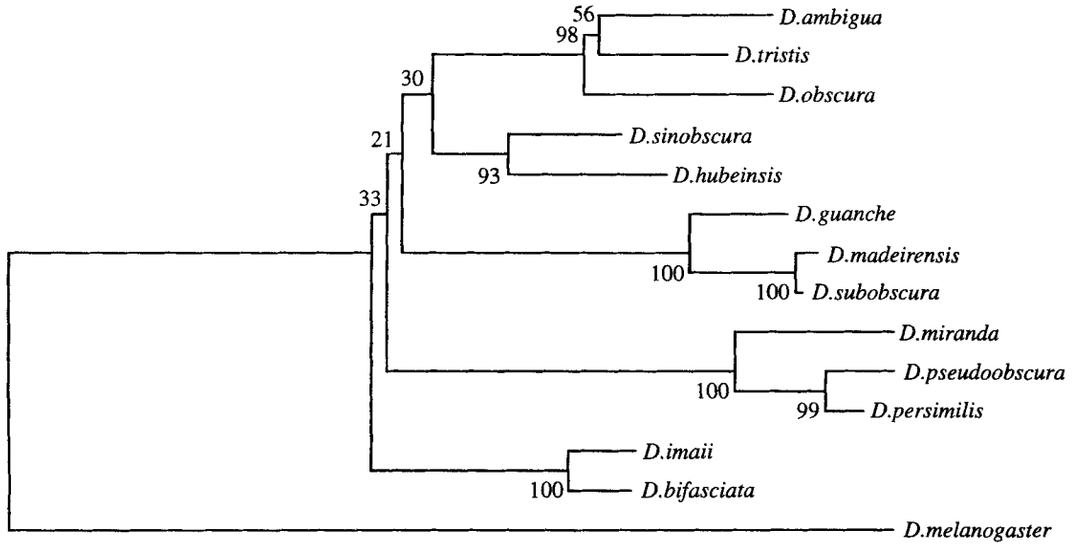

Fig. 4. Unrooted neighbor-joining dendrogram of segments of the *Adh* gene of several *D. obscura* group species. Genetic distance values were calculated according to Kimura (1980). The number at each node represents the bootstrap value per 100 replications

However, another aspect of the *ATOC*180 analysis conflicts with the morphological data. In the neighbor-joining dendrogram (Fig. 2) most sequences of *D. sinobscura* are grouped in a cluster located very far from the sequences of *D. hubeiensis*. Nevertheless, within *D. sinobscura* one sequence (*sin*180/5) is quite different from the other *ATOC*180 repeat units. It shares at several positions characteristic nucleotides not only with the sequences of *D. hubeiensis* but also with those of *D. ambigua* and *D. tristis*. In general, the sequences of *D. hubeiensis* are rather different from those of the other four species. Bachmann and Sperlich (1993) have interpreted the mode of evolution of the *ATOC*180 sequence family as basically gradual. Following their arguments the separation of the *D. hubeiensis* lineage should be regarded as the most ancient cladogenetic event in the phylogeny of the five species. With respect to sequence evolution the exceptional repeat *sin*180/5 must be considered as a member of an ancient branch of the sequence family present in the common ancestor.

Allozyme data as well as nucleotide sequences of 5S DNA repeats and part of the *Adh* gene identify *D. sinobscura* and *D. hubeiensis* as closely related sibling species, but neither of these data sets support without doubt a close relationship of *D. sinobscura* and *D. hubeiensis* to the *D. ambigua*, *D. tristis* and *D. obscura* triad as would be expected from the *ATOC*180 data. *D. sinobscura* and *D. hubeiensis* appear rather as a separate clade within the *D. obscura* group distinctly differentiated from *D. ambigua*, *D. tristis* and *D. obscura*. On the other hand, none of the data sets conflicts basically with the assumptions deduced from the *ATOC*180 sequence trees, locating the *D. sinobscura–D. hubeiensis* clade in the neighbourhood of the *D. ambigua–D. tristis–D. obscura* clade.

A second aspect of this work refers to the taxonomic status of *D. sinobscura* and *D. hubeiensis*, i.e. the question of whether these two *Drosophila* strains are good species and not just geographically isolated and thus slightly differentiated populations of the same species. The observation that flies of both populations can be crossed in both directions, producing fertile male and female offspring (Watabe and Sperlich 1997), is certainly an argument in favour of the one-species hypothesis. This assumption is also supported by the allozyme data. *D. sinobscura* and *D. hubeiensis* differ only at one locus (*Est*-6 in PAGE) and show different polymorphisms at two other loci (*Hk*-3 in

SGE and *Phi* in CAE). Genetic distance values resemble those observed between the European and Japanese strains of *D. bifasciata*.

However, there are other data that support the sibling species concept for *D. sinobscura* and *D. hubeiensis*. First, both species have a different karyotype (Watabe and Sperlich 1997). Second, the *Adh* sequences' divergence matches that found between other closely related species of the *D. obscura* group. Third, the 5S DNA repeat sequences differ even more than those between the closely related sibling species of the *D.* obscura group, *D. subobscura*, *D. madeirensis* and *D. guanche* (Grau and Bachmann 1997). Fourth, in the *D. obscura* species group, the *ATOC*180 satDNA sequences of *D. sinobscura* and *D. hubeiensis* show the highest degree of differentiation. All these observations indicate that almost no gene flow has taken place between *D. sinobscura* and *D. hubeiensis* in nature for a very long period of time. Applying the biological species concept, *D. sinobscura* and *D. hubeiensis* have to be regarded as good, though closely related, morphologically not distinguishable sibling species.

The geographic origin of the *D. obscura* group is still an open question. Some evidence suggests that this group of the subgenus Sophophora emerged in Africa, somewhere in the mountain region of Kenya, whereas another line of arguments points to an East Asian origin. The 'Out of Africa' hypothesis was put forward recently, when five new African species (e.g. *D. microlabis* and *D. kitumensis*) were discovered (Cariou et al. 1988). However, phylogenetic analyses cannot give a definite answer to this question yet. In almost all approaches, the dendrograms revealed clades of closely related species, but the relationships between these clusters could not be resolved with any degree of significance. This is also true for the data sets presented here, as well as for the dendrograms presented by other authors irrespective of the characters used, e.g. mitochondrial RFLPs (Gonzales et al. 1990), or mitochondrial DNA sequences (Gleason et al. 1997). Weak support for the East Asian hypothesis comes from the observations of Acosta et al. (1995). Using two-dimensional SDS protein electrophoresis for cell homogenates from 14 different species of the *D. obscura* group, dendrograms were obtained in which the African species cluster of *D. microlabis* and *D. kitumensis* branch off from all the other lineages at the basis of the tree. The lineages leading to the European and to the American species of the *D. obscura* group separate at similar distances. This is rather in accordance with an Asian origin of the group. Since *D. sinobscura* and *D. hubeiensis* were not included in this study, no final conclusion can be drawn.

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Zusammenfassung

Taxonomische und molekulare Untersuchungen an Drosophila sinobscura und D. hubeiensis, zwei Geschwisterarten der D. obscura – Gruppe

Allozymelektrophorese und drei verschiedene DNA-Sequenzen (*ATOC*180 Satelliten DNA, 5SrDNA Wiederholungseinheiten und Abschnitte des *Adh* Gens) wurden zum Vergleich der nahe verwandten und fertil kreuzbaren ostasiatischen Arten *Drosophila sinobscura* und *D. hubeiensis* verwendet. Die ermittelten Divergenzdaten wurden auch für die Untersuchung der phylogenetischen Beziehungen zu den anderen Arten der *D. obscura* Gruppe herangezogen. Die genetische Divergenzen in der 5SrDNA Wiederholungseinheiten, besonders aber im *Adh*-Gen trennen die beiden Arten eindeutig voneinander und rechtfertigen den Artstatus. Sie sind beide mit den europäischen Arten der D. obscura – Gruppe verwandt, aber das Auftreten von Kopien der für die Artentriade *D. ambigua*, *D. tristis* und *D. obscura* spezifisch und gemeinsamen *ATOC*180 Satelliten-DNA-Familie in der genomischen DNA von *D. sinobscura* und *D. hubeiensis*, stellt diese in deren nächste Verwandtschaft.

References

Acosta, T.; Pinto, F.; Hernandez, M.; Gonzalez, A.M.; Cabrera, V.M.; Larruga, J.M., 1995: Phylogeny of the *Drosophila obscura* group as inferred from one- and two-dimensional protein electrophoresis. J. Zoo. Evol. Research **33,** 101–108.

Ayala, F.J.; Powell, J.R.; Tracey, M.L.; Mourao, C.A.; Perez-Salas, S., 1972: Enzyme variability in the *Drosophila willistoni* group. IV. Genic variation in natural populations of *D. willistoni*. Genetics **70,** 113–139.

Bachmann, L.; Müller, E.; Cariou, M.L.; Sperlich, D., 1992: Cloning and characterization of *KM*190, a specific satellite DNA family of *Drosophila kitumensis* and *D. microlabis*. Gene **120,** 267–269.

Bachmann, L.; Raab, M.; Sperlich, D., 1989: Satellite DNA and speciation: a species specific satellite DNA *of Drosophila guanche*. Z. Zool. Syst. Evolut.-forsch. **27,** 84–93.

–; –; –, 1990: Evolution of a telomere associated satellite DNA sequence in the genome of *Drosophila tristis* and related species. Genetica **83,** 9–16.

–; Sperlich, D., 1993: Gradual evolution of a specific satellite DNA family in *Drosophila ambigua*, *D. tristis* and *D. obscura*. Mol. Biol. Evol. **10,** 647–659.

Cariou, M.L.; Lachaise, D.; Tsacas, L.; Sourdis, J.; Krimbas, C.; Ashburner, M., 1988: New African species in the *Drosophila obscura* species group: genetic variation, differentiation and evolution. Heredity **61,** 73–84.

Davis, B., 1964: Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121,** 404–427.

Eck, R.V.; Dayhoff, M.O., 1966: Atlas of Protein Sequence and Structure. Silver Spring, MD: National Biomedical Research Foundation.

Felger, I; Pinsker, W., 1987: Histone gene transposition in the phylogeny of the *Drosophila obscura* group. Z. Zool. Syst. Evolut.-forsch. **25,** 127–140.

Gleason; J.M.; Caccone, A.; Moriyama, E.N.; White, K.P.; Powell, J.R., 1997: Mitochondrial DNA phylogenies for the *Drosophila obscura* group. Evolution (in press).

Gonzalez, A.M.; Hernandez, M.; Volz, A.; Pestano, J.; Larruga, J.M.; Sperlich, D.; Cabrera, V.M., 1990: Mitochondrial DNA evolution in the *obscura* species subgroup of *Drosophila*. J. Mol. Evol. **31,** 122–131.

Grau, R.; Bachmann, L., 1997: The evolution of intergenic spacers of the 5S DNA genes in the *Drosophila obscura* group: are these sequences suitable for phylogenetic analyses? Biochem. Syst. Ecol. (in press).

Gutknecht, J.; Sperlich, D.; Bachmann, L., 1995: A species specific satellite DNA family of *D. subsilvestris* appearing predominantly in B chromosomes. Chromosoma **103,** 539–544.

Hagemann, S.; Haring, E.; Pinsker, W., 1996: Repeated horizontal transfer of *P* transposons between *Scaptomyza pallida* and *Drosophila bifasciata*. Genetica **98,** 43–51.

Kimura, M., 1980: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Biol. **16,** 111–120.

Kreitman, M., 1983: Nucleotide polymorphism at the *alcohol dehydrogenase* locus of *Drosophila melanogaster*. Nature **304,** 412–417.

Kumar, S.; Tamura, K.; Nei, M., 1993: MEGA: Molecular evolutionary genetics analysis, version 1.0. The Pennsylvania State University, University Park, PA.

Lakovaara, S.; Saura, A.; Lankinen, P.; Pohjola, L.; Lokki, J., 1976: The use of isozymes in tracing evolution and in classifying Drosophilidae. Zoologica Scripta **5,** 173–179.

Marfany, G.; Gonzàlez-Duarte, R., 1991: The *Adh* genomic region of

Drosophila ambigua: evolutionary trends in different species. J. Mol. Evol. **32,** 454–462.

–; –, 1992: Evidence for retrotranscription of protein-coding genes in the *Drosophila subobscura* genome. J. Mol. Evol. **35,** 492–501.

–; –, 1993: Characterization and evolution of the *Adh* genomic region in *Drosophila guanche* and *D. madeirensis*. Mol. Phylogenet. Evol. **2,** 13–22.

Nei, M., 1972: Genetic distance between populations. Amer. Nat. **106,** 283–292.

Preiss, A.; Hartley, D.A.; Artavanis-Tsakonas, S., 1988: Molecular genetics of enhancer of *split*, a gene required for embryonic neural development in *Drosophila*. EMBO J. **12,** 3917–3927.

Saitou, N.; Nei, M., 1987: The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4,** 406–425.

Sanger, F.; Micklen, S.; Coulson, A.R., 1977: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Schaeffer, S.W.; Aquadro, C.F., 1987: Nucleotide sequence of the *Adh* gene region of *Drosophila pseudoobscura*: evolutionary change and evidence for an ancient gene duplication. Genetics **117,** 61–73.

–; Miller, E.L., 1991: Nucleotide sequence analysis of *Adh* genes estimates the time of geographic isolation of the Bogota population of *Drosophila pseudoobscura*. Proc. Natl. Acad. Sci. USA **88,** 6097–6101.

Watabe, H.; Sperlich, D. 1997: *Drosophila hubeiensis*, a new species of the *D. obscura* group from the mainland of China. Jap. J. Ent. (in press).

–; Terasawa, R.; Lin, F., 1996: A new species of the *Drosophila obscura* species-group from China. Jap. J. Ent. **64,** 489–495.

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