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Low allozyme and mtDNA variability in the island endemic species *Drosophila sechellia* (*D. melanogaster* complex)

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Summary. Genetic variability of *D. sechellia* is investigated at both mitochondrial and nuclear levels. The results reveal the existence of a single main type of mtDNA with very few variants and a very low enzyme polymorphism. This situation is consistent with the small population size of this specialized species.
Key words. Genetic variability; mitochondria; allozymes; *Drosophila sechellia.*

Of the drosophilid species so far studied the great majority have high allozyme polymorphism, the average heterozygosity being above 0.10 and sometimes exceeding 0.20[1,2]. There is very little known about the restriction site variability of mitochondrial DNA (mtDNA); however, a large range of nucleotide diversity (an estimate of the average heterozygosity at the nucleotide level[3] (and see below) has been found in the few species studied; from 0.002 up to 0.020[4].

On theoretical grounds the level of polymorphism of both nuclear and mitochondrial genomes should be related to the effective population size, N_e. It is also expected that the population size should be much higher in generalist than in specialist species. The *D. melanogaster* complex, which comprises four species, appears to be a good model for testing such expectations. Two species, *D. melanogaster* and *D. simulans*, are cosmopolitan generalist species with huge populations, both in temperate and tropical regions[5]. The other two species are island endemics, *D. mauritiana* in Mauritius and Rodrigues and *D. sechellia* in the Seychelles. Their ecological niches, however, are different; *D. mauritiana* is a generalist and abundant species found in both natural and domestic habitats[6] while *D. sechellia* is specialized on a single resource, the fruit of *Morinda citrifolia* on a few tiny islands of the Seychelles archipelago about 1000 km northeast of Madagascar[7,8].

The allozyme polymorphism of the two cosmopolitan species has been extensively investigated[9,10] and some data on their mtDNA are also available[4,11,12]. In contrast, the two endemic species are still poorly known. In the present paper the genetic variability of *D. sechellia* is investigated at mitochondrial and nuclear levels and a very low polymorphism is found in both genomes.

MtDNA Restriction site variability. Two samples of *D. sechellia* were collected in the Seychelles in 1981 and 1985. From the first collection on Cousin Island, a mass culture was established. For the second sampling, 27 isofemale lines (26 from Cousin and 1 from Frigate) were established in the field and brought to the laboratory. The mtDNA was analyzed in 22 isofemale lines; 21 from the 1985 sample (20 Cousin and 1 Frigate lines) and one line derived from the 1981 mass culture. MtDNA was extracted from virgin eggs as described in Solignac et al.[13]. Since *D. sechellia* has a very low egg production[8], females were crossed to *D. mauritiana* males and the F1 fertile females were back-crossed each generation to *D. mauritiana* males. After a few generations, each line acquired the high fecundity of *D. mauritiana* although each kept the original *sechellia* mitochondrial genome. MtDNAs were digested with 19 restriction enzymes (see below). Restriction fragments were separated on vertical agarose 1% gels, stained with ethidium bromide and photographed under UV light. Alternatively, restriction

fragments were end-labelled, separated on 1% agarose gels or 6% acrylamide gels, and the dried gels were autoradiographed.

The 22 genomes digested with 19 restriction enzymes produced four different types named *a, b, c,* and *d*. Type *a* is largely predominant and occurs in 18 lines including Cousin 1981 and Frigate 1985 lines and has 93 restriction sites: 10 *Acc*I (the only enzyme used which cuts in the A + T rich region where five sites are present), 6 *Ava*II, 7 *Bcl*I, 3 *Cla*I, 7 *Dde*I, 7 *Eco*RI, 2 *Hae*III, 2 *Hin*cII, (not *Hpa*I), 5 *Hin*dIII, 17 *Hin*fI, 3 *Hpa*I, 3 *H*paII, 6 *Pvu*II, 4 *Sac*I, 3 *Sca*I, 3 *Tha*I, 5 *Xba*I. Type *b*, found in two isofemale lines, is characterized by a single *Ava*I site (all the other lines having no such site). Two additional restriction sites are recorded, a *Cla*I (type *c*) and a *Dde*I (type *d*), each occurring in a single line.

Length variation is also present in the common type (*a*). The three different genomes, short (S), medium (M) and long (L) possess a sequence of 470 bp, including an *Acc*I site, which is repeated 4, 5 and 6 times, respectively[14]. The S and L genomes are found only in heteroplasmic state. Two lines are biplasmic M/L and one triplasmic S/M/L. Length variability is associated with change in the number of *Acc*I sites but this kind of variation has not been taken into account here.

The nucleotide distances[15] between the four mtDNA types are in table 1. The maximum distance value is 0.21%. The nucleotide diversity is the average number of nucleotide differences per site between two randomly chosen DNA sequences[15]. For the whole set (mean in pairwise combinations of the 22 genomes) it is 0.036%. This value is an estimate of the average heterozygosity at the nucleotide level: two mitochondrial genomes taken at random would differ by an average of only 6 or 7 nucleotides.

Table 1. Nucleotide distance matrix for the four mtDNA types described in the text (a–d) in *Drosophila sechellia*. Diagonal gives the number of sites detected in each type, for restriction enzymes which recognize sequences comprising 4, 14/3 (for *Ava*II which has two recognition sequences), 16/3 (for *Acc*I, *Ava*I and *Hin*cII with multiple recognition sequences) and 6 nucleotides respectively, according to table 5.7 in Nei, 1987[3]. The upper half of the matrix gives the number of sites shared by each pair of types. The percentage nucleotide distances, calculated according to formulae 8 and 10 of Nei and Li, 1979[15] are given in the lower half. N = number of lines for each type.

	mtDNA type a N = 18	b N = 2	c N = 1	d N = 1
	32	32	32	32
a	6	6	6	6
	12	12	12	12
	43	43	43	43
		32	32	32
b	0.10	6	6	6
		13	12	12
		43	43	43
			32	32
c	0.10	0.20	6	6
			12	12
			44	43
				33
d	0.11	0.21	0.21	6
				12
				43

This figure differs by one order of magnitude from that of *D. melanogaster* ($\pi = 0.2\%$[11]) and two orders from that of *D. simulans* ($\pi = 1.97\%$[4]). However, in the latter species the comparatively high nucleotide diversity is almost exclusively due to the existence of three well-differentiated geographic types (cytoplasmic races) each exhibiting a very low variability ($\pi = 0.00$ to 0.046[4]).

Allozyme polymorphism. Allozyme polymorphism was studied in both the 1981 (18 to 35 individuals depending on the loci) and 1985 (2 flies for each of the 27 isofemale lines) samples using standard electrophoretic techniques[16]. Thirty-three putative loci were recorded for the following enzymes: phosphoglucomutase (*Pgm*), α glycerophosphate dehydrogenase (α *Gpdh*), fumarase (*Fum*), glucose-6-phosphate dehydrogenase (*G6pdh*), aldolase (*Ald*), aldehyde oxidase (*Ao*), octanol dehydrogenase (*Odh*), alcohol dehydrogenase (*Adh*), esterases (*Estc, Est6, Estp* and two larval loci, *Estl1, Estl2*), superoxide dismutase (*Sod*), sorbitol dehydrogenase (*Sdh*), 6-phosphogluconate dehydrogenase (*6Pgd*), acid phosphatase (*Acph*), xanthine dehydrogenase (*Xdh*), carbonic anhydrase (*Ca*), succinate dehydrogenase (*Su*), hexokinases (*Hk1, Hk2, Hk3*), isocitrate dehydrogenase (*Idh*), malic enzyme (*Me*), malate dehydrogenase (*Mdh*), glutamate oxalate transaminase (*Got*), phosphoglucoisomerase (*Pgi*), glutamate dehydrogenase (*Gdh*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), leucine aminopeptidases (*Lap1, Lap2*) and amylase (*Amy*).

Among the 33 enzymatic loci scored only three, two esterases and the amylase locus, were polymorphic, each with two alleles. Allele frequencies are given in table 2. Interestingly, these three loci are already known to be polymorphic in the three other species in the *melanogaster* complex where, however, they have many more alleles: the average number of alleles per locus is 3.5 for the *Amy* locus, 3.8 for *Estc* and 5.7 for *Est6* in *D. melanogaster*.

Genetic variation based on the same set of 33 loci is compared among the four species of the *D. melanogaster* complex in table 3. There is a clear contrast between *D. sechellia* and its relatives with respect to both the propor-

Table 2. Electrophoretic mobility and allele frequencies at the three polymorphic loci found in *D. sechellia*. *Amylase* mobility is indicated according to Dainou et al.[30]. *Estc* and *Est6* alleles are numbered by comparison to *D. melanogaster* for which allele 100 is the 'fast' one. n = cumulated number of flies investigated from 1981 and 1985 samples.

Locus	Alleles	Frequencies	n
Amy	4.4	0.58	72
	5.4	0.42	
Estc	107	0.87	89
	112	0.13	
Est6	102	0.91	73
	104	0.09	

Table 3. Amount of genetic variation (33 enzymatic loci) in *D. sechellia* and its three closest relatives. For the two cosmopolitan species, only one population is considered here. H: averaged expected heterozygosity; p: proportion of polymorphic loci; N: average number of alleles per locus; n: average number of flies investigated.

Species	H	P%	N	n
D. sechellia	0.027	9	1.09	89
D. mauritiana (Mauritius)	0.218	65	3.15	192
D. simulans (Réunion isl.)	0.159	57	2.06	136
D. melanogaster (Ivory Coast)	0.117	49	1.85	373

tion of polymorphic loci (9% vs 49 to 65%) and the average heterozygosity (2.7% vs 11.7 to 21.8%).

Effective population size: genetic and ecological estimates. It can be reasonably postulated that the island endemic species *D. sechellia* originated from a proto *simulans* population through a severe founder effect. Evidence that actual populations are derived from a few initial founders is provided by the low average number of alleles per locus; it is significantly below that for other species, including *D. mauritiana*, the other island species.

The low mitochondrial and nuclear variability of *D. sechellia* could be a 'remnant' of this founder effect. In Hawaiian *Drosophila*, where several single island endemics are believed to be derived from single successful founders, species exhibit a large range of nuclear heterozygosity, from 0.015 up to 0.128 [17, 18], and some of them have a noticeable level of mtDNA polymorphism [19]. In fact, it has been shown that high levels of heterozygosity should be restored after an extreme bottle-neck [3, 20].

The genetic distance between *D. sechellia* and *D. simulans* is rather high, D = 0.28 [16], a value which does not suggest very recent speciation. Because variability is still low, in both nuclear and mitochondrial genomes, in current populations, genetic drift must have operated after the foundation. Estimates of the effective population size from genetic data could thus be related to long-term evolution of the species rather than to the initial foundation event.

If we assume an evolutionary rate for *Drosophila* mt DNA of $\lambda = 10^{-8}$ [19] and a generation time g = 0.06 years, the effective female population size can be estimated from $N_f = \pi/2\lambda g$ [15] to be about 3.3×10^5. From allozyme data, an average heterozygosity of 0.027 and an average mutation rate per gene per year $v = 10^{-7}$ give an effective population size of 7×10^4 (from $N_e = H/4v(1-H)^3$. The two estimates are not in close agreement. The variabilities of the two genomes are known to have differential sensitivity to demographic bottle-necks [22], but the highest value is obtained for mtDNA, which is the most sensitive to these effects. The discrepancy can probably be explained in part by the inaccuracy of the evolutionary rates used. There is now evidence indicating that mtDNA evolves at similar rates in *Drosophila* [23–25] whereas it evolves 5 to 10 times faster than single copy nuclear DNA in mammals [26].

Accurate ecological estimates of population size for small insects are difficult to obtain. *D. sechellia* is found on some islands of the Seychelles archipelago where its known geographic distribution is limited to about 30 sq. km. Its density could vary from 1000 to 50 000 per sq km, depending on the abundance of the *Morinda* host. *Morinda* produces fruits all year round and *D. sechellia* is not likely to be subject to strong seasonal bottle-necks, so that a reasonable demographic estimate is probably between 10^5 and 10^6.

An agreement between genetic data which reflects long-term evolutionary change and current population size is exceptional. Effective population size estimated in three vertebrate species from molecular distances between mtDNA lineages were two to three orders of magnitude lower than current breeding-population size [27]. For an Australian parrot [28] genetic data suggested a population size of 10^4 whereas direct observations led to an estimate of 10^7. For *D. melanogaster*, Kreitman's [29] estimate of N_e from the *Adh* coding region is only 3.3×10^6. Such discrepancies indicate recent bottle-necks.

In comparison, our estimates of *D. sechellia* lie within a relatively small range and the low genetic variability of the species can be related to its limited geographic distribution and its strict specialization which, together, determine its small and stable effective population size.

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Note added in proof: *D. sechellia* has been recently collected an Mahé island (W. Gehring, pers. comm.).

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Cytogenetic studies of Hynobiidae (Urodela). IX. Karyological characteristics of *Hynobius abei* Sato by means of R- and C-banding

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Summary. Chromosomal characteristics of the salamander species *Hynobius abei*, from Ohimya (Kyoto) were revealed by the techniques of R- and C-banding. The karyotype of *H. abei* was characterized by the shortness of an R-negative (C-positive) band in the terminal region of the long arm of chromosome 2 and a band encompassing the whole short arm of chromosome 10. These two bands in *H. abei* were the shortest among those of the various *Hynobius* species that have been examined. Otherwise no differences could be detected between *H. abei* and seven other pond-type species of *Hynobius* (2n = 56) in terms of the banding patterns of 18 specifically identifiable pairs of their chromosomes.
Key words. Hynobius abei; banding karyotype; R-banding; C-banding.

In order to examine the process of differentiation in the genus *Hynobius* from a cytogenetic perspective, banding analyses of the karyotypes are required for all members of the genus. Since banding analysis was first applied to the chromosomes of salamanders by Kohno et al.[1] (1983, C-banding) and by Kuro-o et al.[2] (1986, R-banding), the banding characteristics of the karyotypes of eight species of the genus *Hynobius* have been described[3–7]. However, for the remaining eight of the 16 *Hynobius* species found in Japan and Korea, including *H. abei*, no information on banded karyotypes is available in the literature.

Hynobius abei is distributed endemically on the Tango peninsula of Kyoto prefecture in Japan where it has been protected since 1983, because the size of the population has decreased significantly. Since the material is hard to obtain, only one report has been published on the chromosome number (2n = 56) and karyological characteristics, excluding banding of the species[8].

Here we report the results of a detailed karyological analysis of *H. abei* performed by R- and C-banding.

Materials and methods

Two egg-sacs of *H. abei* were collected from Ohmiya (Kyoto) with the permission of the Board of Education of Kyoto prefecture. Ten embryos were used for chromosome analysis.

The R-banding (RBA) technique established by Dutrillaux et al. (1973)[9] was used with slight modifications (RBG). Details of the technique have been described elsewhere[2].

For C-banding (CBG), chromosome preparations were made by the method previously used for embryonic cells of *Hynobius*[4], and C-banding was carried out following the method of Sumner (1972)[10].

Results

Karyotype analyses were performed on a total of 48 excellent metaphase spreads from 10 embryos. It was clearly shown that the chromosome number was 56 (2n), which is identical to that given in the report of Seto and Matsui (1984) and also to all the other pond-type species of *Hynobius* studied, with the exception of *H. retardatus*. Banding karyotypes of *H. abei* are shown in figures 1 (R-banding) and 2 (C-banding). Banding analysis allowed 18 out of 28 chromosome pairs to be specifically identified. There are 9 large-sized chromosome pairs (nos. 1–9; meta-, subtelo- and submetacentric), 4 medium-sized chromosome pairs (nos. 10–13; subtelo-, submeta- and metacentric) and 5 pairs of small-sized chro-