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Evolutionary novelties in islands: *Drosophila santomea*, a new *melanogaster* sister species from São Tomé

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The finding of new *melanogaster* sister species may help us in understanding more about how the emergence of genetic novelties, particularly in insular habitats, can result in speciation. Here we report on the discovery of *Drosophila santomea*, which is the first *melanogaster* sibling found off West-equatorial Africa, on São Tomé, one of the Gulf of Guinea islands. Although the eight other *melanogaster* sister species are remarkably conservative in their morphology except for their terminalia, the new find has a morphological trait distinguishing it from all of these: a pure yellow body coloration of both sexes without the normal black abdominal banding. Evidence from the terminalia, polytene and mitotic chromosomes, *period* gene and allozymes are provided indicating that it is nonetheless the nearest relative of *Drosophila yakuba* with which it coexists on the island. The new find is a clear-cut taxon as shown by the production of sterile male hybrids, eventually with developmental defects, in both directions of cross with *yakuba* and by the existence of an altitudinal divide accompanied by a hybrid zone at mid-elevation on the island. Molecular and karyotypic data further support this conclusion. In contrast to the significant divergence of their nuclear DNAs, an intriguing similarity in their cytochrome *b* sequences was observed indicating a recent coalescence common to *santomea*, *yakuba* and also *teissieri* cytoplasms. These were shown to harbour the same *Wolbachia* endosymbiotic bacteria which could possibly be responsible for mitochondrial DNA hitchhiking across the species barrier.

Keywords: *Drosophila santomea*; *melanogaster* subgroup; insular speciation; *period* gene; cytochrome *b* gene; *Wolbachia* surface protein gene

1. INTRODUCTION

(a) *Discovery of a new melanogaster sister species*

The questions of how and how long pre-existing phylogeographical divergences have resulted in extant pairs of sister species (Morritz *et al*. 1992; Avise & Walker 1998; Avise *et al*. 1998) are central to understanding how genetic changes lead to the origin of species (Coyne 1992; Barraclough *et al*. 1998). Sister species of *Drosophila melanogaster* have long ranked among the most important metazoans for speciation (Ashburner 1989; Long & Langley 1993; Coyne & Charlesworth 1997; Powell 1997; Sanchez & Santamaria 1997; Kulathinal & Singh 1998; Ting *et al*. 1998; Sawamura *et al*. 1999) and developmental studies (Halder *et al*. 1995; Wang *et al*. 1996; Gehring 1998; Rutherford & Lindquist 1998). However, with the release of the genomic sequence of *D. melanogaster* it will henceforth become possible to unravel the very functional genomics of a model eukaryotic organism (Ashburner *et al*. 1999; Spradling *et al*. 1999; Adams *et al*. 2000). In that respect *melanogaster* relatives will turn out to play a still more central role as an experimental model in the next few years. However, finding a new *melanogaster* sister species is a rare event. Whenever it has occurred in the past it has invariably given new impetus to the fields of population genetics, developmental genetics and, more generally, evolutionary biology. However, for the last two decades it has been held that no more new siblings could be found in the Afrotropical ancestral home range (but see Wu *et al*. 1995). Here we report on the discovery of a new *melanogaster* sister species, *Drosophila santomea* Lachaise & Harry, sp.n. (short diagnosis below) in the remote, submontane, mist rainforests covering the higher rugged volcanic slopes of São Tomé Island off the Cameroon and Gabon coastlines. This species, which is thought to be endemic in São Tomé, is the first insular *melanogaster* sibling found in the eastern Equatorial Atlantic Ocean and the first insular endemic not belonging to the *simulans* clade. In this paper we present the new species in its insular environment and analyse the reproductive relationships and chromosomal and molecular divergence of the new species with regard to its eight sister species.

(b) *The Cameroon Volcanic Line*

The Cameroon Volcanic Line (CVL) is a south-west–north-east offshore and onshore linear trend of volcanism and uplift extending more than 2000 km from the Guinea Gulf Islands (Annobon, São Tomé, Principé and Bioko) to the Adamawa Plateau in Cameroon. Virtually straddling the Equator, São Tomé Island is one of these high volcanoes (2024 m) and is situated in the oceanic portion of the CVL *ca*. 280 km off the Gabonese coast. The oldest rocks on São Tomé Island are possibly Cretaceous, non-volcanic sandstones or clays; however, the isotopic ages of most volcanic rocks on the island and, more generally, along the CVL fall within 13–15 million years (Myr) ago, even though older dates (Late Oligocene, *ca*. 30 Myr ago) have also been reported (Grunau *et al*. 1975; Lee *et al*. 1994; Meyers *et al*. 1998). The largest volcanic centres are

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in the continental sector and they include Mounts Cameroon (4095 m), Manenguba, Lefo, Bambutos and Oku. The four Gulf of Guinea islands vary greatly in size, altitude and ecology and offer a unique setting for the study of insular speciation. São Tomé (850 km^2) is the most varied of the group and, therefore, it harbours the most characteristic fauna and flora. A variety of plants and animals including Rubiaceae, orchids, begonias, figs, birds and insects are known to have numerous endemic species or subspecies (some possibly shared by Principé). Among the insects is the new species *D. santomea*, one of the 52 species of drosophilids so far recorded on the island (Rocha Pité 1993; D. Lachaise, unpublished).

2. MATERIAL AND METHODS

(a) *Strains and isofemale lines*

A total of 46 isofemale lines of *D. santomea* were founded from wild-caught females originating from the submontane forest of the Obo Natural Reserve on São Tomé Island (collected by D.L. on 7–14 March 1998). Two lines originated from locations below 1300 m including STO.18 at 1140 m. All the other lines were derived from females caught between 1300 and 1450 m. All 46 isofemale lines were tested for *Wolbachia* infection. A total of 21, 12, five, five and two lines were tested for allozymes, polytene chromosomes and the cytochrome *b*, *period* and *Wolbachia wsp* genes, respectively. Five lines, STO.1 and STO.18 which were uninfected by *Wolbachia* (U) and STO.2, STO.7 and STO.12 which were infected by *Wolbachia* (I), were used further in hybridization tests. The isofemale lines used for *Drosophila yakuba* originated from either São Tomé Island (SA), Libreville, coastal Gabon (LBV) or Lopé Forest Reserve, Middle Ogooué, inland Gabon (LO). The lines were chosen so as to include both infected and uninfected lines, if any. A total of 16 (four SA, two LBV and ten LO), three (one SA, one LBV and one LO), five (one SA, one LBV and three LO), two (two SA) and one (one SA) lines were tested for allozymes, polytene chromosomes and the *period*, cytochrome *b* and *wsp* genes, respectively. In *Drosophila teissieri* a total of nine (nine T), five (two B, two T and one CH2), three (three T) and four (two T, one B and one CH2) isofemale lines were tested for allozymes and the cytochrome *b*, *period* and *wsp* genes, respectively. *Drosophila teissieri* T (I) originated from Lopé. The other species tested were represented by isofemale lines originating as follows: *D. melanogaster* LBV.2, 3 and 4 (U) and *Drosophila erecta* e2 (U) from Libreville. All the aforementioned lines originated from the Gulf of Guinea and were established in March–April 1998 except *teissieri* B from Brazzaville, Congo (1989), TNIM from Mount Nimba, Guinea (1976) and CH2 from the Chimanimani Mountains, Zimbabwe (1997). Otherwise, older strains from the Gif stock were used including *D. yakuba*-type strain from Kounden Plateau, Cameroon, 1200 m (Dy115, coll. 1967), *D. simulans* from Usambara, Tanzania (1995) and Seychelles (1981), *Drosophila mauritiana* from Mauritius Island and *Drosophila sechellia* from Cousin Island, Seychelles (Dse 228, 1981). In addition two individuals in alcohol, YLB and YB4 of *D. yakuba* from Tsimbazaza, Madagascar, were tested for cytochrome *b*. YB4 was also tested for *wsp*.

(b) *Hybridizations*

Crosses and backcrosses were performed using five males and five females for each cross and at least five replicates were made per isofemale line pairwise combination and per direction of cross. Virgin partners were confined together from rearing to 15 days post-rearing with mating tubes changed every two days at 21 °C. All the previously cited lines and strains were used.

(c) *Chromosomes and allozymes*

The analysis of the mitotic and polytene chromosomes was performed as in Lemeunier & Ashburner (1984) and the electrophoresis was performed as in Cariou (1987). The 16 loci scored were *Amy*, *Acph-1*, *Acph-2*, *Pgm*, *Adh*, *Gpdh*, *Fu*, *Pgi*, *Est-6*, *Est-c*, *Est-p*, *6-Pgd*, *Xdh*, *Hk-1*, *Hk-2* and *Hk-3*.

(d) Period *and cytochrome* b *genes*

The partial coding region of the *period* gene was amplified using the per5forw (5′-CACCACCGCCAGTAACATAC-3′ starting at position 2598 of the *yakuba* sequence (Thackeray & Kyriacou 1990)) and per6rev (5′-GGAGGAGAAGCTGCTCT-GGG-3′) primers. Direct sequencing was performed using the same primers. The *period* gene sequences reported in this paper have been deposited in the GenBank database (accession no. AF251239–AF251258). The partial cytochrome *b* gene sequence was amplified using the primers CP1 and CP2 and directly sequenced using these two primers and the internal sequencing primer CS2, the orientation of which is similar to that of CP1, as in Harry *et al.* (1998).

(e) *Intracellular endosymbiont sequencing*

Wolbachia 16S rDNA-specific primers, namely 99 and 995 as in Rousset *et al.* (1992), were used to assess the presence of the bacteria in the isofemale lines studied. A fragment of the *wsp* gene (encoding a surface protein of *Wolbachia*) was amplified using primers *wsp*81F and *wsp*691R as in Braig *et al.* (1998). Amplified DNA was sequenced using the primer 691R. The sequences were aligned by hand with those obtained by Zhou *et al.* (1998) between positions 91 and 570.

3. RESULTS

(a) *Morphology*

On the basis of the morphology of the male terminalia the santomean form is a truly full species closely related to *D. yakuba*: there are diagnostic features provided by both the aedeagus and posterior parameres (figure 1). Moreover, the eight *melanogaster* sister species so far known were remarkably similar in their morphology except for their terminalia. A major diagnostic trait defining *melanogaster* subgroup relatives is 'male abdomen black distally'. Therefore, although reminiscent of the sex-linked *yellow* mutant in *D. melanogaster*, the uniquely marked, full yellow colour of the new find (and the lack of colour polymorphism) contrasts with that of the other eight relatives and these characteristics may probably explain why the new species has escaped attention for so long (figure 2).

(b) *Species diagnosis*

D. santomea Lachaise & Harry, sp. n. is close to *D. yakuba* Burla, 1954, but differs in (i) body colour (fully yellow) in both males and females, (ii) the aedeagus and cercus being light yellow instead of dark, (iii) the aedeagal axis making a marked angle with the apodemal axis as against being almost parallel, (iv) the aedeagus being bent apically at an obtuse instead of a right angle, (v) the ventral aedeagal edges being hardly sinuous

instead of markedly sinuous (swan-necked), (vi) the dorsal hooked basis of the aedeagus being significantly larger, (vii) the posterior paramere having a rounded hook instead of the duck-beaked hook, and (viii) the posterior paramere basis not thrown out instead of bellied (figure 1) (a detailed description is in preparation).

(i) *Taxonomy*

We classified our discovery as follows: *Sophophora* subgenus, *melanogaster* group, *melanogaster* subgroup and *yakuba* complex.

(ii) *Etymology*

This refers to the origin of the new taxon from São Tomé Island.

(iii) *Material examined*

For São Tomé Island, all the material types were from one isofemale line (STO.12) from submontane forest at 1400 m in Obo National Park, founding female collected on 8 April 1998 by D.L. A male holotype and five male and five female paratypes are deposited in the Muséum National d'Histoire Naturelle, Paris and five males and five females are deposited in the Natural History Museum in London and United States National Museum in Washington.

(c) *Hybridizations*

In contrast to interspecific crosses between the four *melanogaster* complex species (Lachaise *et al*. 1986; Lemeunier *et al*. 1986) hybridizations between *yakuba* and either *teissieri*, *erecta* or *orena* generally fail (but see Lemeunier *et al*. 1986). The fact that crosses between *santomea* and *yakuba* invariably gave fertile F_1 female and sterile F_1 male hybrids in both directions is therefore unique. Males were sterile but viable. Consistent data were obtained regardless of the geographical origin of the *yakuba* lines used (i.e. São Tomé, coastal or inland Gabon or Cameroon) and regardless of whether or not one or both of the isofemale lines used were infected by *Wolbachia* endosymbionts.

The proportion of crosses which are successful is 0.87 ($n = 46$) in the direction female *yakuba* × male *santomea* as against 0.60 ($n = 45$) in the direction female *santomea* × male *yakuba* ($U = -2.92^{**}$). Moreover, despite great intragroup variance, there was a marked asymmetry in the number of hybrids produced; there were significantly ($t = 3.45^{**}$) more hybrids produced in the cross female *yakuba* × male *santomea* ($n = 46$ and $m_1 = 126.9 \pm 102.2$) than in the reciprocal cross ($n = 31$ and $m_2 = 63.9 \pm 57.5$). *D. santomea* follows Haldane's rule that species hybrids of the heterogametic sex are preferentially sterile or inviable (Coyne 1985; Davis *et al*. 1996).

Disrupting developmental homeostasis is a common effect of interspecific hybridizing (Orr 1990; Khadem & Krimbas 1991; Markow & Ricker 1991). With some of the isofemale lines used here the interspecific crosses generated a number of abnormal phenotypes including mostly severely disorganized abdominal patterning and a diversity of wing defects, some reminiscent of *Notch* in *D. melanogaster*. Moreover, a few hybrid sexual mosaics appeared among the F_1 progeny in both directions of cross. Major defects were significantly ($U = 4.20^{***}$) more numerous in the cross female *yakuba* × male *santomea* (0.018) ($n = 5839$) than in the reciprocal cross (0.005) ($n = 1980$).

The male hybrids generally exhibited a colour phenotype reminiscent of that of their mother, while the female hybrids were generally much more variable. Consistent with what was obtained previously with *yakuba* and *teissieri*, *santomea* females crossed with *D. mauritiana* males produced sterile female and, more rarely, sterile male hybrids. Thus, *mauritiana* males will hybridize with all species of the *melanogaster* subgroup except *orena*. It secondarily appears that, in spite of São Tomé and Mauritius being separated by more than 6000 km, one of the greatest distances known among insular relatives, the two insular endemic species *santomea* and *mauritiana* can produce hybrids, albeit in reduced numbers. Finally and intriguingly, *santomea* males gave rare sterile unisexual female hybrids when crossed with *simulans* females. Consistent data were obtained regardless of the *simulans* female origin (Tanzania or Seychelles). In the last two interspecific crosses, the other direction of cross was unsuccessful. In the no-choice conditions retained here, *santomea* failed to produce hybrids in both directions when crossed not only with *Drosophila orena*, the other sister species endemic in the CVL, but also with *erecta* and *melanogaster* from coastal Gabon, *teissieri* from inland Gabon and *sechellia*, an insular endemic from the Seychelles.

(d) *Mitotic and polytene chromosomes*

The mitotic karyotype of *santomea* is roughly similar to that of *yakuba*, i.e. it has two large metacentric autosomes, a rod-shaped X chromosome of approximately the same size as that of the major autosomal arms, a submetacentric J-shaped Y chromosome and a small chromosome 4. The Y chromosome is somewhat smaller than the X chromosome and almost entirely heterochromatic. However, the two relatives differ in that the *santomea* X chromosome lacks the pericentric heterochromatic band typical of the *yakuba* X chromosome and has a less heterochromatic chromosome 4 (figure 3). The polytene chromosomes of *santomea* are almost homosequential with those of *yakuba*, even for chromosome 4 and none of the 12 *santomea* lines studied was polymorphic. We analysed the chromosomes of all possible female hybrids between several strains of both species. We found only minor differences in the 3R basis around 82E. As usual with *melanogaster* subgroup species, analysing the X chromosome was difficult. There is often extensive ectopic pairing in the middle of the X chromosome within both the *santomea* lines and the *santomea–yakuba* hybrids. The *santomea* X chromosome resembles that of *yakuba* apart from slight differences at its extreme tip.

(e) *Allozymes*

An allozyme survey of 16 loci was performed using 21 *santomea*, 16 *yakuba* and nine *teissieri* isofemale lines. A reference strain was included for the six other species in order to allow the use of previously published data in the phylogenetic analysis (Cariou 1987). Approximately half of the loci were polymorphic in all three species. Five diagnostic loci distinguished *santomea* from *yakuba* (*Amy*, *Pgm*, *Adh*, *Acph-1* and *Est-p*) and six diagnostic loci distinguished *santomea* from *teissieri* (*Amy*, *Fu*, *Acph-1*, *Pgi*,

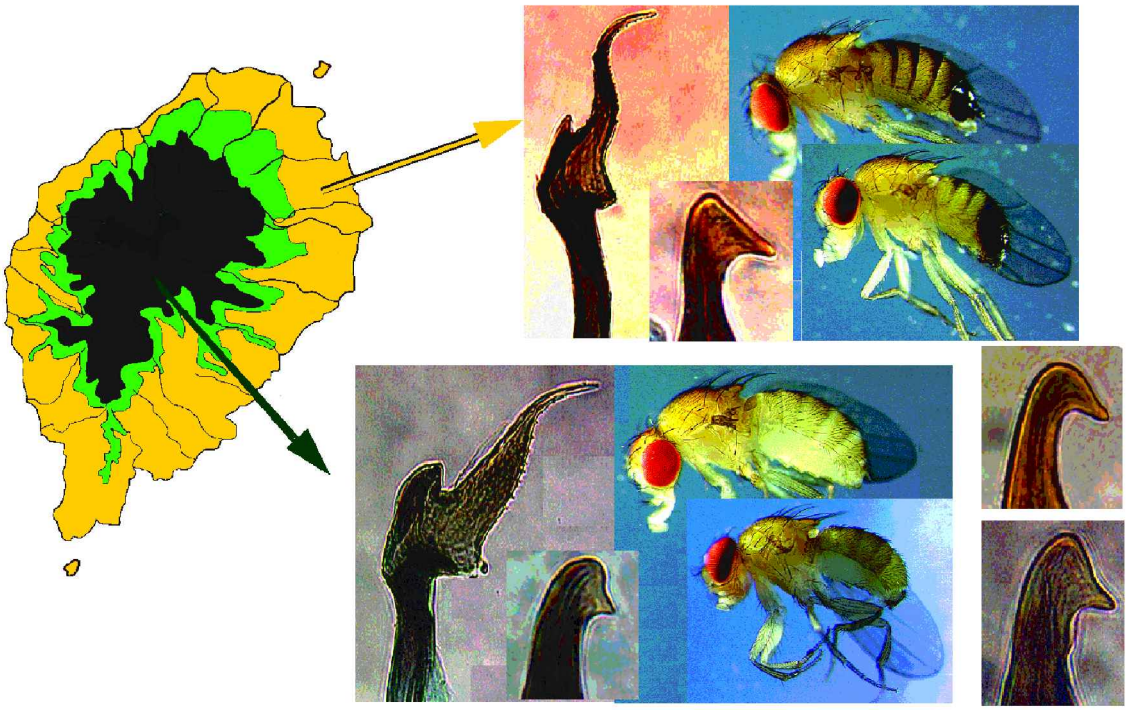

Figure 1. *D. yakuba* (abdomen with dark patterns) and *D. santomea*, sp. n. (full yellow) females (above) and males (below) live sympatrically on São Tomé Island, the former species in lowlands below 1100 m and the latter in highlands above 1100 m (changing colours indicate altitudes: yellow, 0–500 m; light green, 500–1000 m; dark green, 1000–1500 m; black, 1500–2024 m). Among the diagnostic characters which unequivocally distinguish the two sister species are a distinctively shaped aedeagus, swan necked in *yakuba* as against triangular in *santomea* (left insets, height 0.02 mm) and the heads of the posterior parameres, duck beaked in *yakuba* as against rounded in *santomea* (middle insets, height 0.005 mm). The hybrid patterns of the paramere heads are shown in the bottom right insets: female *yakuba* × male *santomea* (above) and female *santomea* × male *yakuba* (below).

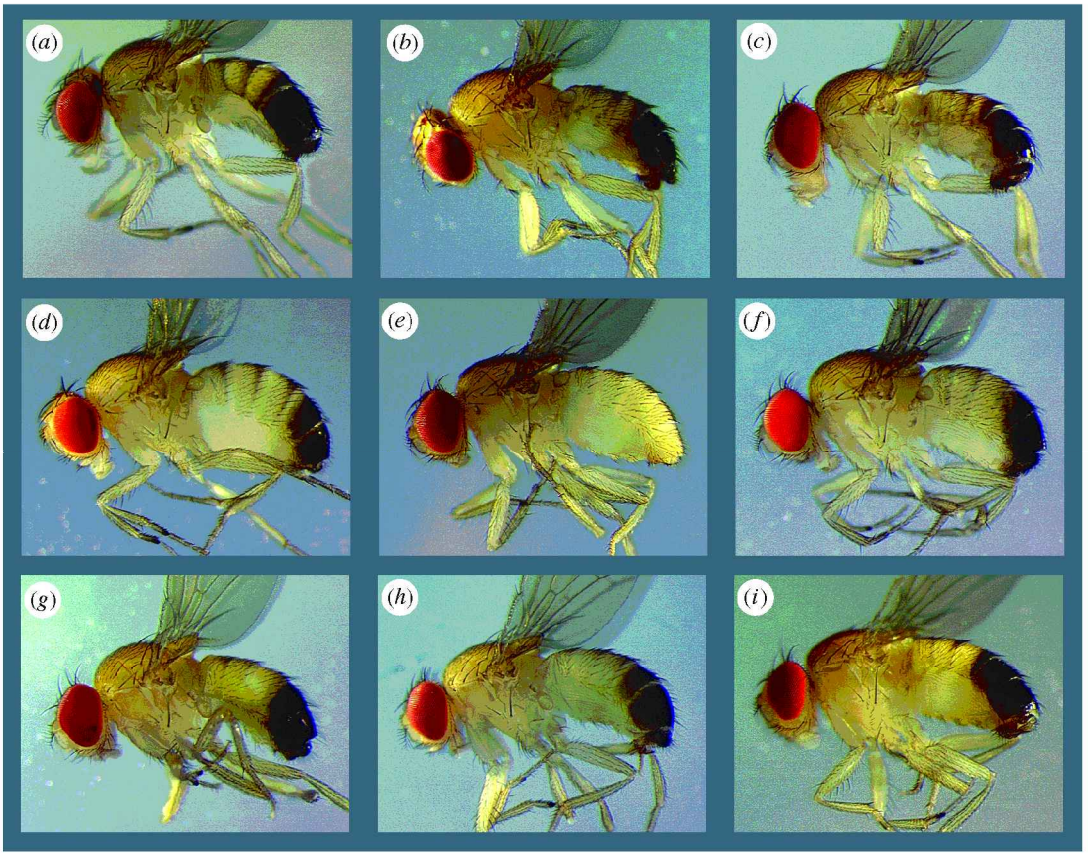

Figure 2. The males of the nine sister species of the *D. melanogaster* species subgroup. The pure yellow coloration without the normal black abdominal banding of the new discovery *D. santomea* distinguishes it from the other eight species. (*a*) *Drosophila sechellia*, (*b*) *D. simulans*, (*c*) *D. mauritiana*, (*d*) *D. yakuba*, (*e*) *D. santomea* sp. n., (*f*) *D. teissieri*, (*g*) *D. orena*, (*h*) *D. erecta* and (*i*) *D. melanogaster*.

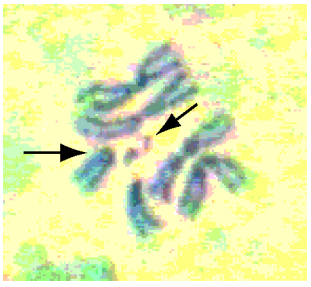

Figure 3. Mitotic chromosomes of a hybrid female from the cross female *santomea* STO.1 × male *yakuba* SA3 show the two diagnostic features affecting chromosomes X and 4 (arrows indicate the *santomea* X and dot fourth chromosomes).

6-Pgd and *Est-p*). This degree of genetic differentiation is similar to that found among the *simulans–sechellia–mauritiana* triad (Cariou 1987). The tree topology (figure 4*a*) indicates the close proximity of *santomea* and *yakuba*.

(f) Period *gene*

We analysed an *ca*. 800 base pair (bp) fragment of the *period* sex-linked gene from six *santomea*, five *yakuba* and three *teissieri* isofemale lines and one strain for the other *melanogaster* subgroup species. The *period* locus is considered as a 'behavioural' gene affecting the organism's biological clock and is therefore, yet controversially, assumed to be a candidate for evolutionary changes in mating behaviour and, hence, possibly in reproductive isolation and speciation (Citri *et al*. 1987; Thackeray & Kyriacou 1990; Peixoto *et al*. 1992; Gleason & Powell 1997). The fragment analysed is part of exon 5 and includes the threonine–glycine (Thr–Gly) repeats known as one of the regions of greatest difference between *Drosophila* species (Citri *et al*. 1987). The nucleotide and protein sequences of the *period* gene were aligned with that of *yakuba* (Thackeray & Kyriacou 1990). A comparison of the *santomea period* gene sequence with that of other *melanogaster* subgroup species revealed patches of conserved and non-conserved sequences. The entire region can be divided into three subregions: the Thr–Gly repeats (amino-acid positions 15–108), a highly conserved region (amino-acid positions 109–183) and another divergent region (amino-acid positions 184–275). As mentioned previously (Citri *et al*. 1987; Thackeray & Kyriacou 1990; Peixoto *et al*. 1992), the number of Thr–Gly repeats varies substantially among *melanogaster* relatives and the lengths we found (figure 4*b*) corroborate those reported by Peixoto *et al*. (1992). Our analysis showed that *santomea* and *yakuba* were identical in having 14 Thr–Gly repeats in all but three of the lines tested: *santomea* STO.4 (which had three additional repeats) and STO.18 and *yakuba* LO.2 where one is deleted, giving these two latter strains the number found in *teissieri*. Also worth noting is that, despite the same number of amino-acid repeats, the *period* gene sequences of the Thr–Gly region at the nucleotide level require both one deletion and one similarly sized insertion events for *santomea* and *yakuba* to be aligned. Such a difference is diagnostic, even though it has no effect on the amino-acid composition.

Moreover, two amino-acid changes appear to be fixed in *santomea* and unique among the *melanogaster* subgroup

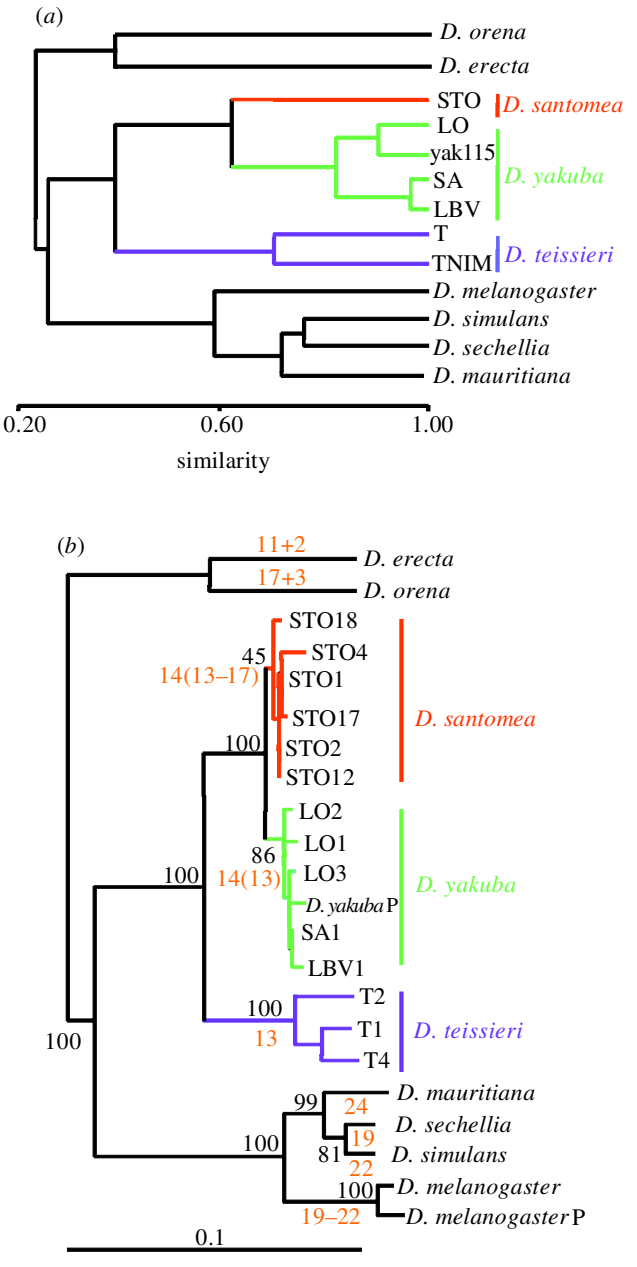

Figure 4. Phylogenetic reconstruction of the *D. melanogaster* subgroup species indicating the close relatedness of *D. santomea* and *D. yakuba*. (*a*) The present data (16 loci) are combined with previously published data for allozymes (Cariou 1987). (*b*) Phylogenetic tree of the *period* sequences derived by the neighbour-joining method with pairwise gap deletions using the Kimura two-parameter distance. Species designations are on the right. The letter P refers to published *period* sequences for *yakuba* (Thackeray & Kyriacou 1990) and *melanogaster* (Citri *et al*. 1987). The numbers on the branches are bootstrap confidence levels (2000 replicates). The number of perfect threonine–glycine repeats is given for each species (orange) and the intraspecific variability observed here is indicated in brackets. Consistent topologies with the tree shown were obtained using either maximum-parsimony (branch-and-bound option with equal weighting of all nucleotide substitutions) or maximum-likelihood (0.5 transition: tranversion ratio with pair gap removal option) methods. All the phylogenetic analyses were made using the programs within the software package SEAVIEW and PHYLO.WIN (Galtier *et al*. 1996). DNA analysis: all sequencing was made using an ABI 373 automated device (Perkin Elmer Applied Biosystems, Inc, The Netherlands).

species: at position 221 a threonine common to all species except *teissieri* is substituted by alanine (T → A). *D. teissieri* is polymorphic for isoleucine (I) or methionine (M). At position 225, a glycine is changed to a valine (G → V). *D. teissieri* is polymorphic for glycine or leucine (L). When all the *period* gene nucleotide sequences are considered, the phylogenetic methods used consistently support the tree shown in figure 4*b*. The *santomea* lines are invariably clustered together and distinct from *yakuba* and *teissieri*. The three main lineages formerly identified among the *melanogaster* species subgroup (Lachaise *et al*. 1988) are also supported by the *period* gene sequences, the *yakuba* complex being closer to the *melanogaster* complex than the *erecta–orena* species pair.

(g) *Cytochrome* b *gene*

The mitochondrial genome was analysed through a sequence of 723 bp of the cytochrome *b* gene from five *santomea*, four *yakuba* and five *teissieri* isofemale lines which were aligned with the *yakuba* reference sequence (Clary & Wolstenholme 1985) between nucleotides 10 656 and 11 378. Out of five sites shown to be polymorphic, two were found in a *teissieri* line (B.12) and two others in the published sequence of *yakuba* (Clary & Wolstenholme 1985). The fifth one separated two groups of haplotypes, both of them including individuals from the three species. Consequently, the difference between haplotypes taken in pairs does not exceed 0.6%. Such a level of similarity confirms the lack of significant polymorphism in the mitochondrial DNAs (mtDNAs) of both *yakuba* and *teissieri* and the negligible differentiation between the two species which was formerly detected (Monnerot *et al*. 1990) by comparing 2000 bp of the control region in two lines and by extensive restriction analyses of the genome of numerous lines with geographical origins encompassing western, eastern and south-eastern Africa. The present data extend the conclusion (between-species similarity) to *santomea*.

(h) *Wolbachia surface protein gene*

This unusually low polymorphism and the similarity of the mtDNAs between species imply a recent and common coalescence time for all haplotypes. A factor likely to clone the mtDNA could be an invasion of the cytoplasm by the maternally inherited intracellular symbiont *Wolbachia* (O'Neill *et al*. 1997; Bourtzis *et al*. 1998; Merçot & Poinsot 1998); we therefore focused our attention on this organism. It was found commonly in the *teissieri* lines (69 out of 72), rarely in *yakuba* (five out of 54) and at an intermediate frequency in *santomea* (13 out of 46). We sequenced part (462 bp) of the hypervariable gene of a surface protein (*wsp*) in those lines analysed for mtDNA which were infected (four *teissieri*, one *yakuba* and two *santomea*). The seven sequences obtained were identical to one another and to that of the Coff Harbour *simulans* analysed by Zhou *et al*. (1998). Moreover, the reduced polymorphism observed on the mtDNA of infected strains was shared by the uninfected ones.

(i) *Hybrid zone*

D. santomea was found in the submontane, mist rainforest between 1200 and 1500 m in the Obo National Park. Although it is still uncertain (albeit plausible) whether *santomea* is present in the highest parts of the mist forest region extending from 1500 to 2024 m in elevation, the species is absent in the lowest cultivated regions of the island where *D. yakuba*, its closest relative, is most abundant (Rocha Pité 1993). Between 1100 and 1250 m the *yakuba*/*santomea* relative abundance ($n = 475$) is 0.67/0.33. Between 1300 and 1450 m ($n = 115$) it shifts to 0.05/0.95. *D. santomea* actually appears dependent on a mist rainforest habitat, whereas *yakuba* can cope with drier, open field habitats. Accordingly, there is a clear-cut difference in altitude between the two siblings, with *yakuba* in areas below 1100 m and *santomea* in highlands above this altitude. There is also a contact zone, possibly a 'hybrid zone' (Barton & Hewitt 1989; Hewitt 1989; Barton & Gale 1993; Butlin 1998) between 1150 and 1450 m in height. There were 11 males with uncertain status among the 601 *santomea* insects captured on the Pico de São Tomé. However, six of these males, which were caught between 1300 and 1430 m, exhibited a clear *santomea–yakuba* hybrid abdominal pattern consistent with those of experimental F_1 hybrids, that is either mixing a yellow epandrium with pale dark patches on the two most posterior tergites and yellow lateral fringes on the last one or a thin T-shaped pattern on a yellow background on the last tergite only. This pattern contrasts with the *santomea* pattern which has remained monomorphic for the full yellow colour over more than 50 generations in the 46 isofemale lines. It also contrasts with the polymorphic patterns exhibited by *D. yakuba*. As expected with a sex-linked gene, our experimental hybrid males display either the *santomea* or the *yakuba period* gene sequence depending on which parental female the X chromosome of the male hybrid came from. Which *period* gene sequence will be found is therefore more or less predictable on the basis of the hybrid male phenotypes. Sequence analysis of the sex-linked *period* gene of four of these natural hybrids showed that three had the *santomea* and one the *yakuba period* gene sequence, thereby indicating that the two sister species can occasionally hybridize in the wild in both directions of cross giving a rough, preliminary overall rate of hybridization close to 0.01.

4. DISCUSSION

(a) *Historical home of the* melanogaster *relative ancestor*

The discovery of *D. santomea* supports the view that, within west central Africa (Lachaise *et al*. 1988), the actual CVL is the presumed historical home of the *melanogaster* sister species' ancestor. The reason is that the CVL is the only place in Africa to harbour both an insular (*D. santomea*) and a mainland (*D. orena*) endemic, the latter living at 2000 m in elevation in the *Syzygium staudtii* submontane forest of Mount Lefo. Moreover, although not restricted to the CVL, *D. erecta* is confined to the Gulf of Guinea coast, its range including and crossing the CVL. Finally, all the relatives which are thought to have arisen from the oldest cladogeneses within the *melanogaster* species subgroup (i.e. *D. orena*, *D. erecta*, *D. teissieri* and *D. yakuba*) happen to coexist in some parts of the mainland CVL. Unlike the Hawaiian archipelago (Carson & Clague 1995) the ages of CVL rocks do not show progressive north-east-directed ageing (Meyers *et al*. 1998) and, therefore, cannot be similarly used in

estimating molecular evolutionary rates by plotting molecular divergences onto K–Ar-based ages (Fleischer *et al*. 1998). At the present stage, it can only be noted that the presumed age of the primeval ancestor of the *melanogaster* species subgroup (Lachaise *et al*. 1988) fits the age (i.e. 13–15 Myr ago) of the paroxysmal phasis of volcanism and uplift all along the CVL quite well.

(b) *Nuclear DNA supports the evolutionary history*

Within the *melanogaster* subgroup, the two nuclear markers used here (allozyme loci and the protein-encoding *period* gene) support both the monophyly of the *yakuba* triad and the claim that *yakuba* and *santomea* are closely related. Whether *santomea* arose from a single invasion of the putative *yakuba* ancestor followed by *in situ* divergence into *santomea* or from a double colonization is still a matter of conjecture. The molecular distinctiveness of *santomea* versus the insular santomean *yakuba* and the lack of increased divergence between this latter and the mainland *yakuba* populations would suggest a double colonization of São Tomé. *D. yakuba* might have colonized the island within the time since the first Portuguese settlement of São Tomé in 1493 and expanded as cultivation proceeded. However, more genetic evidence is needed since the single invasion scenario followed by speciation could be a possibility if there was ongoing gene flow between mainland and insular populations of *yakuba*. Whatever hypothesis is valid, *santomea* most presumably arose from a *yakuba* mainland stock at a time when the geologically old island was entirely covered with forests. However, if both natural selection and random genetic drift can be seen as causes of evolution on islands (Barton 1996), the ecological–altitudinal divide observed between *yakuba* and *santomea* suggests that the full yellow pattern may have been the result of strong selection and possibly rapid adaptation (Carson 1997; Orr & Smith 1998) in the mist forest of São Tomé Island. Although life-history adaptations and reproductive isolation along an altitudinal gradient were formerly observed in grasshoppers (Orr 1996), such an altitudinal hybrid zone between sister species seems quite unique in *Drosophila*.

(c) *Mitochondrial DNA reflects events more recent than the cladogeneses*

Unlike nuclear markers, the subtle differences observed in mtDNA (Monnerot *et al*. 1990) and those which could be assessed in the endosymbiont are irrelevant to the evolutionary history of species. They are definitely more recent than the cladogeneses and can at most reflect the history of the invasion of their cytoplasm by *Wolbachia*. If *Wolbachia* infection accompanied speciation in the *simulans* complex (Rousset & Solignac 1995), propagation of the bacteria occurred after speciations in the *yakuba* complex, indicating that the species barrier can be overcome in spite of hybridization difficulties. The simplest way of interpreting these observations is to assume that a single and recent infection propagated vertically to the three species. We think this event would have occurred only once because it is unlikely that three independent and almost contemporaneous infections by the same bacterial strain would have taken place. And, even if this had happened, different and, hence, specific haplotypes would have been cloned. We assume that the event was recent because noticeable mtDNA polymorphism from the ancestral haplotype would otherwise have been restored in the three species. The mtDNA of those flies which are currently not infected has also been cloned by the same event, suggesting that the infection has been secondarily lost. This interpretation implies that the micro-organism propagated to the three species a long time after their speciation, generating mtDNA hitchhiking. Therefore, unlike nuclear genes, mtDNA is unhelpful in tracing back the speciation events within the *yakuba* triad.

5. CONCLUSION

The existence of the diagnostic features of male terminalia, i.e. the production of sterile F_1 hybrid males sometimes accompanied by severe developmental defects and subtle differences in mitotic chromosomes but marked genetic and molecular divergence of the nuclear DNA of the yellow new insular established population, consistently show that there are valid and decisive reasons for assigning species rank to it. Otherwise, in spite of its distinctive colour, all the data including male terminalia resemblance, the production of fertile F_1 female hybrids, homosequential polytene chromosomes and evidence through nuclear DNA of common ancestry conclusively show that *santomea* is the nearest relative of *yakuba* and must therefore be placed in the *yakuba* complex within the *melanogaster* species subgroup. It is all the more intriguing that the presumably derived species (*santomea*) hybridizes more easily with distantly related sister species (*mauritiana* and *simulans*) than with the taxon (*teissieri*) which arose more directly from the putative ancestral species, yet similar cases are known in the *obscura* group (Powell & DeSalle 1995). It could be that the ability of *santomea* to hybridize is a homoplasy (i.e. a reversion). The new species provides a case example where the nuclear genome mirrors the evolutionary history of species while the mtDNA is irrelevant to it, indicating further (Morritz *et al*. 1992) that mtDNA alone should not be used without corroboration from other evidence for inferring species boundaries. Aside from the cytoplasmic compartment, all the data provide compelling evidence that insular speciation of a new *melanogaster* sister species occurred in the Gulf of Guinea. As has long been emphasized by Carson (1997) for Hawaiian *Drosophila*, the differentiation of three insular *melanogaster* sister species, namely *D. mauritiana*, *D. sechellia* (Coyne 1989; Coyne & Charlesworth 1997) and now *D. santomea*, in three different Afrotropical oceanic archipelagoes indicate that islands are most suitable for gene pool 'refashioning' occurring.

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