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Divergence between *Drosophila santomea* and allopatric or sympatric populations of *D. yakuba* using paralogous amylase genes and migration scenarios along the Cameroon volcanic line

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Abstract

We have used two paralogous genes (*Amyrel* and *Amy*) of the amylase multigene family to reconstruct the phylogeny of the nine *Drosophila melanogaster* subgroup sister species, including *D. santomea*, the newly discovered endemic from São Tomé island. The evolutionary divergence of these genes is of special interest as it is suspected to result from physiological evolution via gene duplication. This paper describes the relationship between the geographical origin of the various strains and the patterns of mating and phylogeny, focusing on the evolution of *D. santomea* and its relationship to other species and their niches. The *Amyrel* and *Amy* data indicate that, contrary to expectations, the sympatric insular *D. yakuba* population is less closely related to *D. santomea* than allopatric mainland ones, suggesting that the extant insular *D. yakuba* population on São Tomé results from a recent secondary colonization. Data for sympatric and allopatric *D. yakuba* suggest that *D. santomea* arose from a mainland *D. yakuba* parental stock when montane habitats of the Cameroon volcanic line extended to lower altitudes during colder and less humid periods. Despite their different modes of evolution and different functions, the *Amyrel* and *Amy* genes provide remarkably consistent topologies and hence reflect the same history, that of the species.

Keywords: amylase multigene family, *Drosophila santomea*, *D. yakuba*, Gulf of Guinea, phylogeny, São Tomé

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Introduction

The molecular evolution of multigene families has recently received considerable attention (Liao 1999; Kogan *et al*. 2000). It has been argued that reconstructing the evolution of multigene families is central to understanding the evolutionary meaning of duplication and divergence of genes as a result of physiological adaptation through gene duplication (Da Lage *et al*. 1998). This study was carried out to verify the extent to which the evolutionary patterns of homologous (paralogous) genes of a multigene family mirror the phylogeny of sister species.

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The amylase gene is well suited to studies on evolution of multigene families and for testing how adaptation and speciation are relevant to each other. The amylase gene (*Amy*) has been studied in a number of *Drosophila* species and a duplicate structure was found in *D. melanogaster* and all the related species within the subgroup (Boer & Hickey 1986; Daïnou *et al*. 1987; Shibata & Yamazaki 1995). The number and arrangement of the genes, two close copies divergently transcribed, are conserved throughout the subgroup (Payant *et al*. 1988; Shibata & Yamazaki 1995).

Amyrel, a paralogous gene of the amylase family (so-called because it is 'Amylase-related') was recently identified in *D. melanogaster* and other species of the *Sophophora* subgenus, *D. ananassae* and *D. pseudoobscura* (Da Lage *et al*. 1998). Unlike *Amy*, *Amyrel* is a single copy gene, which has a specific intron position and is physically distinct from the *Amy* cluster on chromosome 2R (*Amy* maps at 54A and

Amyrel at 53D). But although the two genes are closely set, they are strikingly different from one another because their divergence is 40% at both the nucleotide and the amino acid levels. The function of *Amyrel* remains unknown. However, in contrast to the *Amy* genes, *Amyrel* is transcribed in the larvae of *D. melanogaster* but not in adults, suggesting that the *Amyrel* gene is akin to the breeding sites used. *Amyrel* is thought to evolve approximately twice as fast as *Amy* in the various species studied by Da Lage *et al*. (1998). These authors, therefore, suggested that this gene could result from a duplication of *Amy* followed by accelerated and selected divergence toward a new function.

We have now carried out a comparative analysis of *Amy* and *Amyrel* in a pair of sister species, one of which is suspected to have arisen through strong selection and possibly rapid adaptation. Our analysis has focused on a presumably suitable pair of sister species of *Drosophila*, *D. yakuba* and *D. santomea*, which are two *melanogaster* siblings of the afrotropical *yakuba* complex within the *melanogaster* species subgroup.

D. yakuba is widely distributed throughout the tropical African mainland from the Sahel to Swaziland and has spread to inland Madagascar and the neighbouring Ste-Marie island to the east of Tamatave, Zanzibar island off Tanzania in the Indian Ocean, and São Tomé Island off Gabon in the Atlantic Ocean. Its capacity to colonize new insular environments is probably related to its ability to cope with open, even semiarid habitats. *D. santomea* is a newly discovered insular species endemic to São Tomé, one of the four Gulf of Guinea islands, and the nearest relative of *D. yakuba* (Lachaise *et al*. 2000). Both siblings occur on the São Tomé volcano, but they are segregated because they live at different altitudes. *D. yakuba* inhabits lowland secondary habitats and *D. santomea* the montane mist forest above 1100 m. The two species come into contact at that elevation and form a hybrid zone. Whether this altitude divide is due to a change in elevation (that is to climatic change) or to a dramatic change in the vegetation type is still controversial. However, given the great elevational range of *D. yakuba*, from sea level to 3000 m in East Africa, for instance on Mt Elgon in Kenya (Lachaise *et al*. 1988), it appears that *D. yakuba* is not limited by elevation but by the unsuitable mist forest habitat. The confinement of *D. santomea* to higher altitudes could likewise be more due to the suitability of the type of vegetation (the mist *Podocarpus* forest) than to elevation *per se*.

The speciation of *D. santomea* from a mainland or insular *D. yakuba* stock may, therefore, be the result of adaptation to new resources in the submontane mist forest. If so, and considering that one gene (*Amyrel*) is only expressed in larvae while *Amy* is expressed in both larvae and adults, we anticipate that there should be some differences in the evolutionary patterns of these paralogous genes in the two *Drosophila* sister species. We tested this prediction by sequencing the *Amy* and *Amyrel* genes, which have different patterns of evolution (Da Lage *et al*. 1998), for the new species *D. santomea* and the other eight sister species of the *melanogaster* subgroup. We analysed *Amy* and *Amyrel* in the remote insular population of *D. santomea* and in a diversity of sympatric (insular) and allopatric (mainland) populations of *D. yakuba*, all originating from the Gulf of Guinea. Our purpose was to compare gene sequences from between and within species to infer the phylogenetic relationships of *D. santomea* and make comparisons to the phylogeny previously derived from the 'clock' gene, *period* which is located on the X chromosome. A long series of between-species hybridization tests was carried out using sympatric and allopatric strains to assess further the species identity and affinity. Finally, migration scenarios that might account for the origin of *D. santomea* are discussed.

Materials and methods

Samples in the Gulf of Guinea

Samples of the isofemale lines of the three siblings of the *yakuba* complex (*Drosophila santomea*, *D. yakuba* and *D. teissieri*) was obtained by one of us (D.L.) in March–April 1998 in the Gulf of Guinea in central West Africa, with a special focus on the offshore portion of the Cameroon volcanic line (CVL) more especially São Tomé. Table 1 summarizes the origin (location, country), the habitats and resources where the foundresses of the isofemale lines were caught. The references of the lines used are indicated. Molecular data were based on six *D. santomea* isofemale lines from one population (São Tomé) and five *D. yakuba* isofemale lines from four populations (one insular, São Tomé, and three continental) from the Gulf of Guinea (Fig. 1).

Origin of the other melanogaster *sister species*

We used the other seven sister species of the *melanogaster* subgroup to reconstruct the phylogeny. We retained one population line from the Gulf of Guinea whenever possible, except for the two Indian Ocean endemics. Thus, we used one isofemale line (the type line 154.1) of *D. erecta* from Lamto in the Ivory Coast, where it breeds exclusively in the syncarps of at least three species of *Pandanus*. We also analysed *D. orena*, which was recorded only once in one location (at Bafut N'Guemba at 2100 m elevation) and has, therefore, long been considered to be endemic to the *Syzygium* submontane forest of Mt Lefo on the Bamiléké Plateau in western Cameroon (Lachaise *et al*. 1988). *D. orena* would then be endemic to one volcano of CVL, and *D. santomea* and *D. orena* can be seen as the insular and mainland counterparts of CVL endemics. No additional isofemale lines have ever been collected since the discovery of *D. orena*

Table 1 Origins of the isofemale lines used

Drosophila	date	Origin	Altitude	Country	Habitat/Resource	isofemale lines
santomea	1998	São Tomé island	1100 m –1500 m	São Tomé e Príncipe	mist montane forest *Ficus chlamydocarpa fernandesiana* (Moraceae)	STO.1, 2, 3, 4, 5, 6, 7, 9, 11, 12, 30, 37
yakuba	1998	São Tomé island	1100 m	São Tomé e Príncipe	secondary forest	SA.1, 2, 3, 4
yakuba	1998	Libreville	sea level	Gabon	man-modified habitat	LBV.1, 2
yakuba	1998	Lopé	500 m	Gabon	Marantaceae forest	LO.1, 2, 3, 4, 5, 6, 7, 8, 9, 10
yakuba	1967	Kounden Plateau	1100 m	Cameroon	savanna-forest mosaic	Y115
teissieri	1998	Lopé	500 m	Gabon	Marantaceae forest *Duboscia macrocarpa* (Tiliaceae)	T.2
teissieri	1971	Chirinda	1100 m	Zimbabwe (Mt Silinda)	montane rainforest	128.2
erecta	1971	Lamto	200 m	Ivory Coast	riverine swamps *Pandanus* spp. (Pandanaceae)	154.1
orena	1975	Bafut N'Guemba	2100 m	Cameroon (Mt Lefo)	*Syzygium staudtii* (Myrtaceae) submontane forest host plant unknown	188.1
sechellia		Cousin island	sea level	Seychelles	island shoreline *Morinda citrifolia* (Rubiaceae)	
simulans		unknown				
mauritiana		Mauritius island	sea level	Mauritius		
melanogaster		Canton-S		Stock		

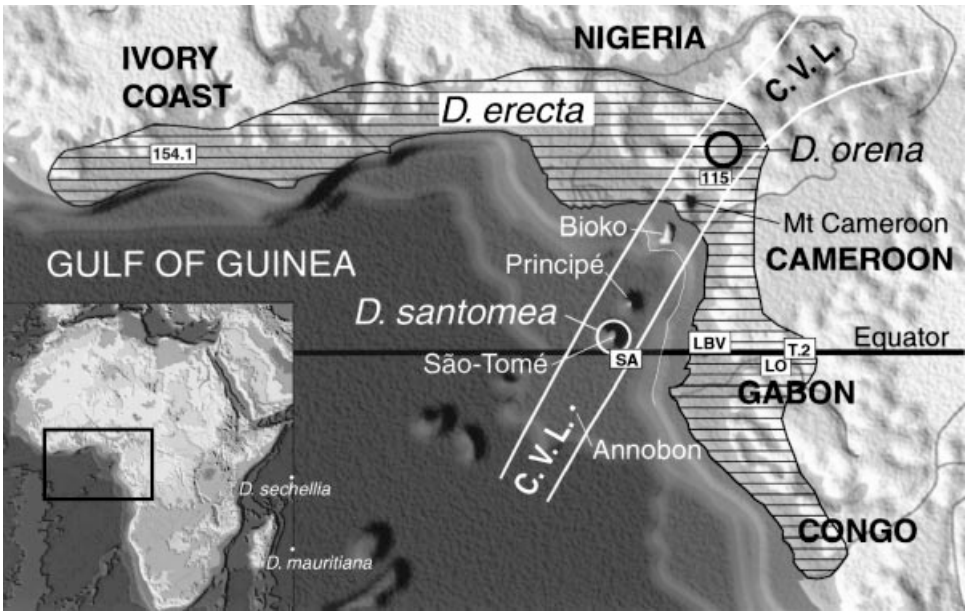

Fig. 1 Distribution of the *melanogaster* siblings endemic to west-west central Africa. Two of them are confined to a single volcano in the Cameroon volcanic line (CVL): *Drosophila orena* on the mainland Mt Lefo, *D. santomea* on São Tomé island. The distribution of the *Pandanus*-breeding *D. erecta* is hatched. The labels represent the sampled populations of *D. yakuba* (LO: Lopé; LBV: Libreville; SA: São Tomé), *D. teissieri* (T.2: Lopé) and *D. erecta* (154.1: Lamto). São Tomé is at the crossroads between the equator and CVL, a 1600 km-long chain of volcanic centres extending north-east from Annobon island in the Atlantic Ocean to the Biu and Ngaoundéré Plateaux (Fitton & Dunlop 1985; Lee *et al*. 1994). Although the CVL has been active for some 65 Myr, there are 12 volcanic centres, whose ages range from 30 Myr to the present (Halliday *et al*. 1990; Meyers *et al*. 1998). Potassium-Argon (K-Ar) datings have suggested that the Adamawa uplift and the islands of São Tomé, Príncipe and Bioko were formed during a synchronous phase of crust uplift and volcanic activity in the Miocene (Grunau *et al*. 1975; Meyers & Rosendahl 1991).

in 1975. As a result, all published data on that species are based on the single (188.1) isofemale line founded at the time of the finding of the species. Finally, we included in the analysis the *D. yakuba* Y115 type strain (one isofemale line) which originated (1967) from Mangoum near Foumbot at 1100 m on the Kounden Plateau, part of the Bamiléké Plateau (Bamenda-Banso block) that is within CVL.

Outside the Gulf of Guinea, we used the type strain (128.2) of *D. teissieri* which originates from the high Chirinda forest reserve at the top of Mt Silinda in south-eastern Zimbabwe near the border with Mozambique at an elevation of 1100–1200 m (H.E. Paterson, personal communication, 26th May, 1977), one strain of the three species of the *simulans* clade originating from the Indian Ocean; *D. sechellia* from Cousin islands in the Seychelles, *D. mauritiana* from Mauritius island, and *D. simulans* of unknown origin. *D. melanogaster* Canton-S was also added.

Hybridization tests

Two series of interspecific crosses were made using either multifemale strains (pools) or isofemale lines (iso ♀). The *D. santomea* pool was started by mixing 50 females and 50 males of each of 10 isofemale lines (STO.1, 2, 3, 4, 5, 6, 7, 9, 11, 12). The *D. yakuba* São Tomé pool was made from a mix of 20 females and 20 males from each of four isofemale lines (SA.1, 2, 3, 4). The *D. yakuba* pool from Libreville, coastal Gabon was made by mixing 20 females and 20 males from each of two isofemale lines (LBV.1, 2). The *D. yakuba* pool from Lopé, Central Gabon was made by mixing 50 females and 50 males from each of 10 isofemale lines (LO.1, 2, 3, 4, 5, 6, 7, 8, 9, 10).

A second series of crosses was made using isofemale lines separately. A total of 10 (STO.1, 2, 3, 4, 5, 7, 11, 12, 30, 37) and 12 (*idem plus* STO.9, STO.16) isofemale lines from the same population were used for *D. santomea* females and males, respectively. Four isofemale lines from one sympatric (SYM.) insular population (SA.1, 2, 3, 4) and five isofemale lines from three allopatric (ALLOP.) mainland populations, LBV (2), LO (2), 115 (1) of *D. yakuba*, were used.

The fertility of the male and female hybrids was tested by backcrossing approximately 20 per cent of a total of 565 crosses.

Analyses of Amyrel *and* Amy

D. santomea amylase genes were amplified using primers designed from published sequences of *D. yakuba* and *D. teissieri* (Shibata & Yamazaki 1995) to match conserved sequences at the 5′ and 3′ flanking regions. The 1526 bp

fragment amplified included the full coding *Amy* sequence: forward primer 5′CAGAGTGAAACTGAACTTCC3′, reverse primer 5′CCAGCTGTTTACAACTTGGC3′. Single fly DNA extracts were amplified using standard polymerase chain reaction (PCR) conditions and the following thermal cycler conditions: 3 min at 94 °C; 30 cycles of 30 s at 94 °C, 1 min at 57–58 °C and 2 min at 72 °C; followed by a final 10 min at 72 °C. As two genes have been reported for all species of the *melanogaster* subgroup, the PCR products were purified and cloned in a PGEM-T cloning vector (Promega). Three to five clones were sequenced for each individual from each isofemale line.

The primers for the *Amyrel* locus were designed to match the conserved 5′ and 3′ flanking regions reported by Da Lage *et al*. (1998): forward primer 5′TCGTAAATTGGACCCAAGCG3′; reverse primer 5′CATACATTATGTGCGTTCG3′. The method used for *Amyrel* was similar to that of *Amy* with the following thermal cycler conditions: 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 1 min at 55 °C and 2 min 30 s at 65 °C; followed by a final 10 min incubation at 72 °C. The PCR products were also cloned in the PGEM-T cloning vector before sequencing. The 1597 bp fragment amplified included the full *Amyrel* gene.

All of the sequencing was performed by the dye terminator chemistry using an ABI 373 sequencer (Perkin Elmer Applied Biosystems Inc.). The sequences obtained from this work are available in GenBank (accession nos for *Amy*: AF280880–280885 *santomea*, D17737 *yakuba*-dist, D17738 *yakuba* prox, AF280889 *yakuba* LO, AF280888 *yakuba*-LBV, AF280886–280887 *yakuba*-SA, AF280890 *teissieri* T2, D17735 *teissieri*-dist, D17736 *teissieri*-prox, X04569 *melanogaster* 1 prox, D17734 *simulans*-prox, D17730 *mauritiana*-prox, D17732 *sechellia*-prox, D17728 *erecta*-prox, D21129 *orena* prox; and for *Amyrel*: AF280868–280873 *santomea*, AF039561 *yakuba* Y115, AF280878 *yakuba* LO, AF280876–280877 *yakuba*-LBV, AF280874–280875 *yakuba*-SA, AF039557 *teissieri*, U69607 and AF022713 *melanogaster*, U96159 *simulans*, U96157 *mauritiana*, AF039558 *sechellia*, AF039562 *erecta*, U96158 *orena*).

Data analysis

Sequence data from the nine *D. melanogaster* subgroup species were analysed with SEQAPP for Macintosh (Gilbert 1992) and aligned with CLUSTAL W (Thompson *et al*. 1994). Reconstruction of phylogenetic trees was based on the full coding sequence of *Amy* (1482 bp) and *Amyrel* (1479 bp) and derived by either distance (neighbour-joining, NJ) or maximum likelihood (ML) methods. The NJ reconstructions used pairwise evolutionary distances estimated using the Kimura 2-parameter (K2P) (Kimura 1980) and the Tajima and Nei (TN) model of substitutions (Tajima & Nei 1984), as implemented in PHYLO_WIN (Galtier *et al*. 1996). Bootstrapping was based on 2000 replicates. For ML tree inference we used the Tamura and Nei (TrN) (Tamura & Nei 1993) substitution model from the TREE-PUZZLE 4.02 program (Strimmer & Von Haeseler 2000). Quartet puzzling support values were based on 2000 iterations. *D. orena* was used as an outgroup in both *Amyrel* and *Amy* phylogenetic trees. Using such a related outgroup is possible considering that more distant outgroups like *D. ananassae*, a distantly allied species of the *melanogaster* group (Lemeunier *et al*. 1986), provided topologies where *D. orena* consistently occupies a basal position with regard to the remaining *D. melanogaster* subgroup species. There is also considerable evidence that *D. orena* arose from the most ancient split within the *melanogaster* species subgroup (Lemeunier *et al*. 1986; Cariou 1987; Lachaise *et al*. 1988; Russo *et al*. 1995). Published sequences from proximal (prox) and/or distal (dist) *Amy* genes (Shibata & Yamazaki 1995) were also used. Among the *Amy* genes we were not able to distinguish between proximal and distal copies. However, the sequence divergence between such closely linked copies is generally very low due to concerted evolution (Brown *et al*. 1990; Popadic & Anderson 1995; Shibata & Yamazaki 1995), making the tree topology not very sensitive to the use of one duplicate or the other.

Results

Hybridizations

Table 2 summarizes our present knowledge of the reproductive relationships between the nine sister species of the *melanogaster* subgroup, including *Drosophila santomea*. Although the basic data implicating *D. santomea* have been published (Lachaise *et al*. 2000), they are given here as further evidence on a larger scale. Table 2 highlights the novelty of the reproductive pattern of *D. santomea*, which offers the possibility of producing fertile female hybrids in both directions of cross between two sister species of the *melanogaster* subgroup outside the *melanogaster* complex.

The number of successful interspecies and intraspecies crosses as against the number of crosses were tested (N_s/N_t) between *D. santomea* from São Tomé island and either sympatric insular or allopatric mainland (Gabon, Cameroon) populations of *D. yakuba*. The 'success' of hybridizations was assessed by the production of female plus male F_1 hybrids regardless of whether they were sterile or fertile. Crosses between isofemale lines involving *D. santomea* females and *D. yakuba* males gave a similar proportion of successful crosses regardless of whether the population of the latter species was sympatric or allopatric with that of the former species (29/57 vs. 26/46, $U = -0.571$, $P = 0.284$, NS). Similarly, crosses in the other direction gave results that were irrelevant to the origin of the *D. yakuba* population (213/268 vs. 210/263, $U = 0.848$, $P = 0.198$, NS).

Table 2 Reproductive relationships between the nine *Drosophila* sister species of the *melanogaster* species subgroup including *D. santomea*, the new endemics from São Tomé island, in the Gulf of Guinea

	yakuba complex				*melanogaster* complex		*simulans* clade		
Drosophila ♀ ♂	*orena*	*erecta*	*teissieri*	*yakuba*	*santomea*	*melanogaster*	*sechellia*	*simulans*	*mauritiana*
orena	+	no F_1	no F_1	no F_1	no F_1	no F_1	no F_1	no F_1	no F_1
erecta	no F_1	+	no F_1	no F_1	no F_1	no F_1	no F_1	no F_1	no F_1 ♂ ster. F_1 ♀
teissieri	no F_1	no F_1	+	no F_1	no F_1	no F_1	no F_1	no F_1	ster. F_1 ♂ ster. F_1 ♀
yakuba	no F_1	no F_1	no F_1	+	**ster. F_1** ♂ **fert. F_1** ♀	no F_1	no F_1	no F_1	ster. F_1 ♂ ster. F_1 ♀
santomea	no F_1	no F_1	no F_1	**ster. F_1** ♂ **fert. F_1** ♀	+	no F_1	no F_1	no F_1	ster. F_1 ♂ ster. F_1 ♀
melanogaster	no F_1	no F_1	no F_1	no F_1	no F_1	+	no F_1 ♂ ster. F_1 ♀	no F_1 ♂ ster. F_1 ♀	no F_1 ♂ ster. F_1 ♂
sechellia	no F_1	no F_1	no F_1	no F_1	no F_1	ster. F_1 ♂ no F_1 ♀	+	no F_1	**ster. F_1** ♂ **fert. F_1** ♀
simulans	no F_1	no F_1	no F_1	no F_1	no F_1 ♂ ster. F_1 ♀	ster. F_1 ♂ no F_1 ♀	**ster. F_1** ♂ **fert. F_1** ♀	+	**ster. F_1** ♂ **fert. F_1** ♀
mauritiana	no F_1	no F_1	no F_1	no F_1	no F_1	ster. F_1 ♂ no F_1 ♀	**ster. F_1** ♂ **fert. F_1** ♀	**ster. F_1** ♂ **fert. F_1** ♀	+

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1        10         20         30         40         50         60
GTGAGTGGTT GCCTTCTCCA GTGAAGAACG ATTGGCTAAT GGTAT---TT TACTCCGCAG santomea
.........T .......A.. ...A...... ...G...... ...CC---.. .A........ yakuba LO/LBV
.........T ..T....A.. ...A...... ...G...... ...CC---.. .A........ yakuba SA
.........T ..C ...... ...AA..... ...G...... ...CT---.. .A........ yakuba Y115
.........T ..C....... ...TG..... T..G...... ...CT---.C .A........ teissieri
.........C .......T.. .GTT...... ...T...... ...CC---.C .A........ teissieri T2
......T-.A .....TC... .CTT...... ...A.T.... ...GT---.. .TT....... melanogaster
......A..A .A...TC... ..TT...... ...A.T.... ..CGTG--.. .TT....... simulans
......T..A .....TC... ..TT...... ...A.T.... ...GTT--.. .TT...A... mauritiana
......T..A .........G .C.T.....T G..AA..... ...GC---.. .T..TT.... sechellia
.......C.T .......... ...T.....C ...G.T.... ...CT---.. .T.A...... erecta
.........T ..C......C ...T...... ...G...... ...CTTTT.C .T.A...... orena
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Fig. 2 Sequence alignment with introns of *Amyrel* genes of *Drosophila santomea* and other related species of the *D. melanogaster* subgroup.

The fertility of the male and female interspecific hybrids was tested by backcrosses with some of the various F_1 progenies — and invariably gave sterile F_1 males and fertile F_1 females in both directions of cross. F_1 hybrids between sympatric and allopatric *D. yakuba* were fertile.

As a consequence, there was no difference in the postzygotic isolation between *D. santomea* and sympatric or allopatric populations of *D. yakuba*. In contrast, when all *D. yakuba* populations were pooled [*D. santomea* ♀ × *D. yakuba* ♂ (55/103) as against *D. yakuba* ♀ × *D. santomea* ♂ (423/531)] there was a significant difference in the success of hybridization, depending on the direction of cross ($U = -5.663$, $P = 0.00$***).

Amy *and* Amyrel *genes*

The amylase gene of *D. santomea* has the same structure as that of all the other species. The gene is 1482 bp long, has no intron and the putative protein is 494 amino acids (aa) long. The *Amyrel* of *D. santomea* has the typical structure of this gene. There is a short intron (57 bp) at position 655, the coding sequence is 1479 bp long and the putative protein has 493 aa. This structure is conserved for all the species within the *melanogaster* subgroup, yet the length of the intron varies from 57 to 60 bp.

For both *Amy* and *Amyrel*, there was greater variation in synonymous sites than replacements and for *Amyrel* greater variation in introns than in exons, as expected under a hypothesis of reduced constraint, although we analysed a limited sample of isofemale lines for the three species *D. santomea*, *D. yakuba* and *D. teissieri*.

The six *Amyrel* introns of *D. santomea* have identical sequences, but they are differentiated from the other species. Their alignement (Fig. 2) separates the intron of *D. santomea* from that of *D. yakuba* including the Y115 reference strain.

The three species, *D. santomea*, *D. yakuba* and *D. teissieri*, showed a similar trend in that Amyrel protein appeared to

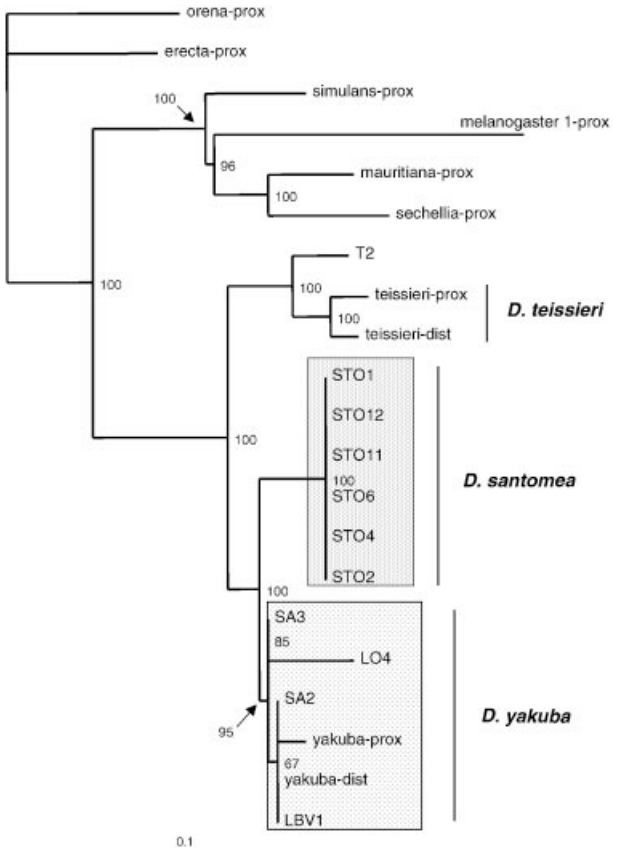

Fig. 3 Phylogenetic reconstruction of *Amy* sequences from *D. santomea* and *D. yakuba* from the Gulf of Guinea and their seven sister species of the *D. melanogaster* subgroup. The tree was constructed by maximum likelihood (ML) (ts/tv = 2; Tamura and Nei model of substitutions; -ln likelihood = 3229) as implemented in PUZZLE 4.02 (Strimmer & Von Haeseler 2000). Quartet puzzling support values are based on 2000 iterations. Species designations are on the right. Sequences from proximal (prox) and/or distal (dist) genes are from Shibata & Yamazaki (1995).

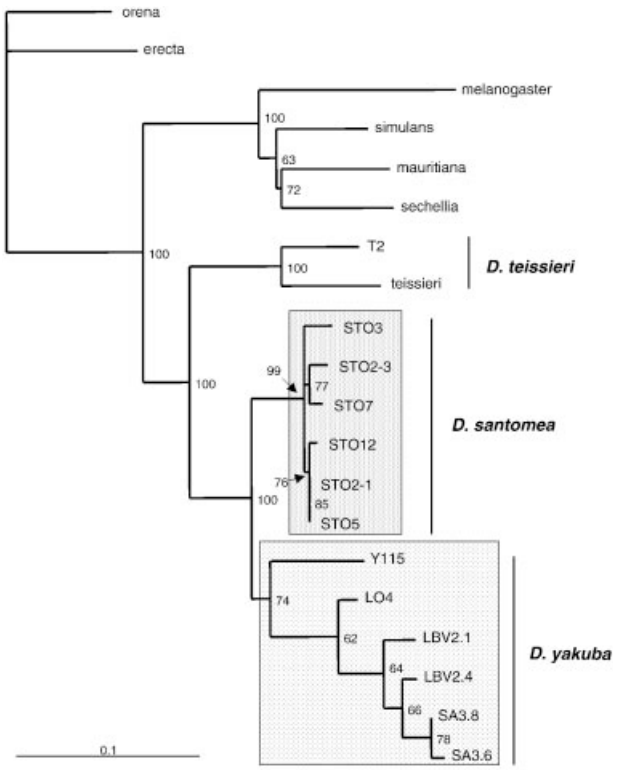

Fig. 4 Phylogenetic reconstruction of *Amyrel* sequences from *Drosophila santomea* and *D. yakuba* from the Gulf of Guinea and their seven sister species of the *D. melanogaster* subgroup. The tree was constructed by maximum likelihood (ML), as in Fig. 3 (–ln likelihood = 4376). The sequence of *D. melanogaster* is from Da Lage *et al*. (1998).

vary more within species than Amy. The *Amy* genes of the *D. santomea* strains had identical amino acid sequences and only one site was polymorphic for the *Amyrel* gene. However, the amylase protein differed much more between *D. santomea* and its eight relatives than AMYREL, as there were eight replacements, five of which were unique among the *melanogaster* subgroup species. The glutamine at position 163, which is common to all species, was replaced by glutamic acid (Q → E). The arginine at position 173 was changed to histidine (R → H) — this domain is involved in calcium binding. The lysine at position 322 was replaced by glutamine (K → Q), the threonine at 353 was changed to proline (T → P), and the arginine at 422 was replaced by a leucine (R → L). Moreover, the change from tyrosine to histidine (Y → H) at position 321 occurred in both *D. santomea* and *D. orena*. Three of these changes were electrically neutral and the three others correspond to loss of positive charges, which explains the peculiar electrophoretic mobility of the *santomea* amylase variant. We found that *D. santomea* had a fast variant with a mobility similar to that of AMY1 of *D. melanogaster* and very distinct from those in *D. yakuba* and *D. teissieri*, which both had two electromorphs; AMY 3.4 is predominant in *D. teissieri* and AMY 4.4 in *D. yakuba* (Daïnou *et al*. 1987).

Gene tree estimates

Figures 3 & 4 show the ML reconstruction of the phylogenetic tree of the nine *D. melanogaster* subgroup species, including six *D. santomea* and five *D. yakuba* isofemale lines from the Gulf of Guinea, based on the 21 nucleotidic sequences of the *Amy* genes and the 20 sequences of *Amyrel*. We obtained the same overall topology from the two genes, *Amy* and *Amyrel*, regardless of the method (ML or NJ). The Quartet puzzling support values were very consistent with the NJ bootstrap values. All the nodes on the gene trees had high quartet puzzling support values

close or equal to the maximum, except for the node leading to *D. yakuba*, indicating a clear gene differentiation between species. The sequence data based on *Amy* and *Amyrel* supported the present species designation and relationships. All the species of the *D. melanogaster* complex were clustered together and their positions were in agreement with the generally admitted relationships: *D. erecta* was near *D. orena* and the four *melanogaster* siblings were clustered together, giving further evidence of the validity of the *Amyrel* and *Amy* trees. There was, however, one exception — the position of *D. melanogaster* branching within the *simulans* clade in the *Amy* tree.

The sequences of *D. santomea*, *D. yakuba* and *D. teissieri* samples clustered with their respective groups and each of these groups had no *Amyrel* or *Amy* sequences from the other groups.

Apart from these intraspecies groupings, the trees always supported sister taxa relationships among species. In particular, *D. santomea* consistently formed a sister group to *D. yakuba*. *D. teissieri* also appeared to be a sister group to a clade that included the two former species for both *Amy* and *Amyrel* genes, confirming the reality of the *yakuba* species complex.

The tree topologies for *D. yakuba* suggested different patterns. The *Amy* tree supported substantial amounts of gene flow within the species, as there does not appear to be a tendency for the different lines to cluster according to their geographical origin. Instead, the *D. yakuba* isofemale lines or strains were interspersed (Fig. 3). It should however, be noted that the *Amy* gene evolves more slowly than *Amyrel* and the lower number of phylogenetic informative sites within *D. yakuba* makes the *Amy* tree less reliable at the intraspecific level.

The *Amyrel* tree showed the opposite tendency, with a good agreement with geographical affinities. The *D. yakuba* Y115 line from the mainland CVL (Kounden Plateau, West Cameroon) was in the most basal position, followed by the line from inland Gabon, the two lines from coastal Gabon and then, terminally, the two lines from São Tomé island (Fig. 3). Although weakly supported (boostrap value = 60%), this Y115 line clustered with the *D. santomea* group in the NJ construction (tree not shown). Given the terminal positions in the *D. yakuba* cluster of both SA lines, these two *D. yakuba* lines from the insular saotomean population did not appear to be the closest to the *D. santomea* cluster, but the most recently evolved lines.

Discussion

Further evidence for Drosophila santomea *as a good species*

Large scale crosses between *Drosophila santomea* and *D. yakuba* invariably produce sterile male and fertile female hybrids, with a significant difference in the success rates of hybridization depending on the direction of cross. This supports our earlier findings (Lachaise *et al*. 2000). Although premating isolation tests were not performed, these data suggest that there is a marked asymmetry in behavioural isolation between the two siblings. There has been considerable debate as to whether such asymmetry indicates the direction of evolution. The initial hypothesis of Kaneshiro (1976) was that the larger ancestral *D. yakuba* population retained the more elaborate mate recognition system and discriminated against *D. santomea* males with the simplified system, whereas females with the simplified requirements accepted equally readily both derived and ancestral males. However, our crosses were definitely easier between *D. yakuba* females and *D. santomea* males than vice versa. Which supports the contrary prediction of Watanabe & Kawanishi (1979) that ancestral species show less premating isolation from derived species than vice versa (see Powell 1997 pp. 240–241 for review and references therein). Nevertheless, we still do not know how long the allegedly derived species (*D. santomea*) has been isolated from the mainland stock (*D. yakuba*). We agree with Powell (1997) that any such asymmetry resulting from simplified mate recognition systems in derived populations is presumably temporary. New complex systems probably arise and this may be a major event in speciation.

The sterility of male hybrids definitely indicates that the two forms are distinct yet closely related species. And the two paralogous genes (*Amy* and *Amyrel*) also support the validity of *D. santomea* as a good species, the sister species relationship between *D. santomea* and *D. yakuba*, and the monophyly of the *D. santomea*, *D. yakuba* and *D. teissieri* group, whichever method is used to construct the phylogenetic trees. These data are fully consistent with those obtained with the *period* gene and a range of other data, including the morphology of terminalia, chromosomes and allozymes (Lachaise *et al*. 2000). Thus, at least three nuclear genes on various chromosomes, *Amy* and *Amyrel* on chromosome 2, and *period* on the X chromosome, provide consistent evidence that *D. santomea* and *D. yakuba* are a pair of siblings within the *yakuba* species complex, which is invariably identified as a monophyletic group.

Two endemic siblings in the CVL

The finding of *D. santomea* in São Tomé island indicates that the CVL is the historical home range of the *melanogaster* species subgroup. Two of the 12 volcanic centres of the Cameroon line are now known to harbour one endemic *melanogaster* sibling. *D. orena* is confined to one volcano (Mt Lefo) of the continental sector of CVL and *D. santomea* to one volcano of the offshore sector of CVL. Both live in fragmented submontane forest patches, and CVL as a whole, therefore, appears to be an archipelago made up of

oceanic and continental rainforest 'islands'. These findings emphasize the role of specific montane areas as evolutionary centres (Fjeldsa & Lovett 1997; Cobb *et al*. 2000). Both *Drosophila* endemics are confined to remote altitudinal submontane forests. Even *D. erecta*, which is commonly found in lowland *Pandanus* swamps, has been found at 2100 m in the CVL, together with *D. orena*, *D. teissieri* and *D. yakuba*, at Bafut N'Guemba on Mt Lefo (Lachaise *et al*. 1988). Therefore, CVL was probably the major corridor involved in the speciation of *melanogaster* sister species. A similar phenomenon occurred more recently among the *simulans* clade through the Indian Ocean archipelagos.

Sympatric vs. allopatric D. yakuba

We used a diversity of *D. yakuba* lines from São Tomé island (i.e. the offshore CVL) and various neighbouring places of the Gulf of Guinea mainland in an attempt to determine which *D. yakuba* parental stock gave rise to *D. santomea*. We tested *D. yakuba* lines from coastal Gabon and inland Gabon and one line from the continental sector of CVL (Kounden Plateau in West Cameroon). Assuming that speciation of *D. santomea* resulted from evolution on the São Tomé volcano, there should be less molecular divergence between *D. santomea* and sympatric *D. yakuba* than between *D. santomea* and any allopatric *D. yakuba*. There should also be greater divergence between the insular *D. yakuba* population and any mainland ones than between the mainland populations. Our data for the *Amyrel* and *Amy* genes clearly show that *D. santomea* is not more closely related to the sympatric *D. yakuba* population than to any continental one. On the contrary, the *Amyrel* phylogenetic tree indicates that the insular (SA) *D. yakuba* lines branch distally rather than basally within the *D. yakuba* cluster. Thus, contrary to expectation, they appear to be the most distantly related *D. yakuba* lines with regard to *D. santomea*, suggesting that the extant insular *D. yakuba* population on São Tomé results from a very recent secondary colonization.

In agreement with this, there is no difference in the postzygotic isolation between *D. santomea* and sympatric or allopatric populations of *D. yakuba*. But it is still possible that the prezygotic behavioural isolation with sympatric *D. yakuba*, especially in the hybrid zone, was reinforced; this is currently being tested. Taking into account the above arguments, and if reinforcement is to be expected in such a case, then the secondary colonization of São Tomé by *D. yakuba* may not have had enough time for any such reinforcement to evolve.

This poses the question of where *D. santomea* arose, assuming double colonization. Although the evidence is still weak, both the *Amyrel* and *Amy* phylogenetic trees point to the onshore portion of CVL. The *D. yakuba* (Y115) line from the Kounden Plateau, within the larger Bamiléké Plateau in western Cameroon, which was used for the *Amyrel* tree, branches in a very basal position among the *D. yakuba* lines, regardless of the algorithm used. ML indicates that Y115 clusters with the Gabonese and Saotomean *D. yakuba* lines, but as an outlier. NJ enhances this tendency, and puts Y115 outside the *D. yakuba* species-specific cluster, but its place between the *D. yakuba* and the *D. santomea* clusters is poorly supported (low boostrap value of 60), whereas the bootstrap is near 100 for all the other clusterings.

Such lineage sorting indicates that some forms within the *D. yakuba* gene pool have kept some ancestral characters common to *D. yakuba* and *D. santomea*. Another possibility is that the branching of *D. yakuba* lines, especially the reference strain Y115, also corresponds to a pattern expected if the sequences of *D. yakuba* and *D. santomea* have histories that include some recombination events. This single *D. yakuba* line from mainland CVL is at least an indication where populations with ancestral traits could be found if they still exist. We are currently analysing DNA sequence variations in *D. santomea*, *D. yakuba* and *D. teissieri* to determine more precisely the amount and pattern of variation, as these estimates reflect the histories of populations. There are also some ecological arguments suggesting that the *D. yakuba-santomea* ancestor had opportunities to migrate through CVL.

The Podocarpus *migration route*

The stepping-stone colonization of a mainland *D. yakuba-santomea* ancestor into the Gulf of Guinea islands may have been coincident with the spread of *Podocarpus* during some colder and less humid periods in oxygen isotope stages 5d and 5b (Frédoux 1994). This occurred around 150 000–130 000 years ago (Van Andel & Tzedakis 1996), when the African rainforest became fragmented and montane habitats extended to lower altitudes (Maley 1987). Frédoux (1994) stated that the abundance of *Podocarpus* pollen during that cooling period in deep-sea cores taken in the Gulf of Guinea suggests there was a broad geographical distribution of this conifer in western Africa. That abundance is in contrast to its present-day restriction to montane habitats of CVL between 800 and 2000 m and São Tomé Island above 1400 m. Its wide occurrence during the transitional phases of cooling was favoured by coolings in the equatorial zone (Maley *et al*. 1990). The very restricted species ranges of the two montane endemics, *D. orena* and *D. santomea*, match quite nicely two such scattered fragmented ranges of *Podocarpus*. The CVL shown in Fig. 1 could be the route by which *Podocarpus* pollen, and also the *D. yakuba-santomea* ancestor, were transported to the Gulf of Guinea islands. The tropical mountains, which are cooler than the lowlands, with mists replacing rain (Maley

1987), may have favoured the speciation of *D. santomea*. However, there is still no evidence of an association between *D. santomea* and *Podocarpus*. An alternative pathway would be the fig tree route.

The Ficus chlamydocarpa *migration route*

D. santomea has so far been found only on fallen figs of the hemiepiphyte fig tree, *Ficus chlamydocarpa*, at 1200–1400 m in the São Tomé submontane forest. This fig tree species is endemic to CVL, where there are three subspecies (Berg & Wiebes 1992). *F. chlamydocarpa* ssp. *chlamydocarpa* is found in the submontane forests of Cameroon and Bioko, *F. chlamydocarpa* ssp. *fernandesiana* (Berg 1988) occurs in São Tomé at 340–1400 m (Lejoly 1995; D. Lachaise, personal communication), and *F. chlamydocarpa* ssp. *latifolia* (Berg 1988) grows in Annobon (Pagalu) (15 km^2) at low altitudes. The mainland fig tree subspecies is rare, growing only in a few submontane forests at altitudes of 1300–2000 m. It has been recorded in the following localities: 40 km east-south-east Nkambe; Mt Tabenken, 10 km south-east Nkambe; Dschang, Mts Bambutos near Dschang, Mben, Fotouni, 20 km north-north-east Bafang, Mt Cameroon, Tonja (Berg *et al*. 1985). These locations are on either side (north-east and south-west) of Bafut N'Guemba on Mt Lefo where *D. yakuba* was found up to 2400 m, and the mainland subspecies of this fig tree happens to occur at 2000 m. Hence, the *D. santomea*/*D. yakuba* split could have occurred at the same time as, and be related to, *F. chlamydocarpa* subspeciation.

Whichever host plant is implicated, there seem to have been repeated opportunities for *D. yakuba* to spread along the CVL. However, while the fragmentation of the host-plant range and hence of the *D. yakuba-santomea* ancestor home range is plausible for the mainland CVL and Bioko, it is not for São Tomé.

The island of Bioko, formerly Fernando Po, lies on the continental shelf 32 km off the coast of Cameroon, and is therefore part of the continental sector including Mount Cameroon (4005 m). Bioko is separated from the shore of Cameroon by a strait only 60 m deep. Data from cores off the west coast of Africa, which record changes in pollen flux from the continent, indicate that the sea level was 70 m below the present level some 39 000–36 000 years ago during the oxygen isotope Stage 3 (van Andel & Tzedakis 1996). The last glacial aridity maximum occurred some 22 000–13 000 Carbon 14 (^{14}C) years ago (approximately 23 000–14 500 'real' years ago). Bioko became an island only after a postglacial rise in sea-level around 13 000–12 000 years ago. This largest (800 km^2) and highest (2850 m) island of the four Gulf of Guinea islands, is still unexplored for *Drosophila* and could well harbour a *D. yakuba* population from which *D. santomea* might have arisen, possibly by a founder effect.

The other three islands, Annobon, São Tomé and Príncipe, were probably never connected to the African mainland or to each other. Consequently, the colonization of São Tomé by the *D. yakuba-santomea* ancestor requires a long migration of 280 km from the Gabon coastline. If it came from the Cameroon mainland through the CVL it would require a stepwise 220 km leap from Bioko to Príncipe and then a 146-km one from Príncipe to São Tomé. The colonization of Príncipe from the mainland would also require airborne transport over 220 km.

Any attempt to determine the time of the split between *D. yakuba* and *D. santomea* must take into account these palaeobiogeographic and molecular clock data. However, the use of molecular clocks requires independent estimates from one or two former sources to calibrate the clock (Powell 1997). There are no reliable estimates of the rates of substitution in *Amyrel*, which is evolving faster than *Amy* (Da Lage *et al*. 1998). A synonymous rate of 6.8×10^{-8} substitutions per nucleotide per year has been proposed for the distal amylase duplicate of *Amy* (E.N. Moriyama unpublished, in Li 1997). We applied this to the synonymous site divergence (K2P distance) and estimated that the split between *D. santomea* and *D. yakuba* occurred some 0.45 Ma. This absolute time of divergence is not too inconsistent with the *Podocarpus* pathway scenario (considering all the uncertainty of molecular clock calibration, it could be within the same order of magnitude), and may possibly match the time required for *F. chlamydocarpa* subspeciation.

In conclusion, although the products of the two genes of the amylase family studied differ markedly, and the genes have evolved differently and are differently expressed, there is, nonetheless, an overall consistency within their phylogenetic patterns. Both genes may have been implicated in the same adaptation toward new resources in the submontane forests of the CVL. These resources could be *Podocarpus* or any *Podocarpus*-associated plant at higher altitudes or some *Ficus* at lower altitudes. The many amino acid replacements in the amylase protein of *D. santomea* may indicate resource specialization. We, therefore, postulate that the evolutionary changes occurring at both the *Amyrel* and the *Amy* loci have some common feature that is relevant to the adaptation of *D. santomea* to local resources

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This paper is a contribution to a broad study on speciation and molecular polymorphism in insular and continental sister species of the *Drosophila melanogaster* subgroup in the Afrotropical region. Marie-Louise Cariou is Head of the Laboratory 'Populations, Genetics & Evolution', of the CNRS at Gif-sur-Yvette, and has a broad interest in gene evolution and molecular polymorphism. Jean-François Silvain is from the Institut de Recherche pour le Développement (IRD) and is deeply involved in molecular phylogeny and ecology, and plant-insect relationships. Vincent Daubin is a PhD student and has contributed the data on the amylase multigene study. Jean-Luc Da Lage is a geneticist at the CNRS and is especially interested in the molecular genetics and evolution of multigene families. Daniel Lachaise is an evolutionary ecologist at the CNRS and has a special focus on speciation of Afrotropical *Drosophila*. He discovered *D. santomea* in São Tomé Island.
