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Incomplete sexual isolation in sympatry between subspecies of the butterfly *Danaus chrysippus* (L.) and the creation of a hybrid zone

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Subspecies *chrysippus*, *dorippus* and *alcippus* of the butterfly *Danaus chrysippus* differ at three biallelic colour gene loci. They have partially vicariant distributions, but their ranges overlap over a substantial part of central and East Africa, where hybridism is commonplace. We now report that the West African subspecies *alcippus* differs from other subspecies, not only in nuclear genotype but also in mitochondrial haplotype in both allopatry and sympatry. The maintenance of concordant nuclear and cytoplasmic genetic differences in sympatry, and in the face of hybridisation, is *prima facie* evidence for sexual isolation. Other evidence that suggests *alcippus* may be isolated from *chrysippus* and *dorippus* include differences in sex ratio (SR), heterozygote deficiency at one site and deduced differences in patterns of migration. We suggest that, within the hybrid zone, differential infection of subspecies by a male-killing *Spiroplasma* bacterium causes SR differences that restrict female choice, triggering rounds of heterotypic mating and consequent heterozygote excess that is largely confined to females. The absence of these phenomena from hybrid populations that test negative for *Spiroplasma* supports the hypothesis. The incomplete sexual isolation and partial vicariance of *alcippus* suggests that it is a nascent species.
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Introduction

Danaus chrysippus is a widespread and abundant butterfly that is distributed throughout the Old World tropics in open habitats. In Africa, the species is divided into three largely vicariant subspecies (Talbot, 1943) (Figure 1), *alcippus* in the western Sahel, south of the Sahara Desert, *dorippus* in the north-east (northern Kenya and Somalia), and *chrysippus* (formerly known as *aegyptius*) in the remaining areas (Owen, 1971; Pierre, 1973; Smith *et al*, 1997, 1998). We have examined over 15 000 specimens of *D. chrysippus* in European museum collections (Smith *et al*, 1998) and find that the geographical relation of *alcippus* and *chrysippus* (Figure 1) differs profoundly between the two sides of Africa. In the west, there is a clean boundary around 2°N that separates the two subspecies, on either side of which hybrids are very rare; *alcippus* alone occupies West Africa from Senegal to the Camaroun–Gabon border, whence south to the Cape *chrysippus* is the only form. Even collections from the islands of Fernando Po (off Camaroun) and Saõ Thomé (west of Gabon) are also monomorphic for *alcippus* and *chrysippus*, respectively. On the other hand, moving east across the continent towards Ethiopia and Kenya, collections reflect morph-ratio clines, with *alcippus* being steadily replaced, to the north-east by *dorippus* and by *chrysippus* to the south-east. The biogeographical evidence suggests that, in the west, equatorial populations of *chrysippus* and *alcippus* are parapatric and isolated, whereas, in the east *chrysippus* and *dorippus* overlap extensively with one another and with *alcippus*.

While the partial vicariance of the three subspecies probably reflects past geographical isolation, in Uganda, southern Kenya, Tanzania and parts of several neighbouring countries, but rarely elsewhere, two or more interbreeding polymorphic forms, that match in phenotype their respective subspecies and share their names, are found in sympatry and hybridise (Owen and Chanter, 1968; Smith, 1975a, 1980; Gordon, 1984). The polymorphism is unique among the 157 species of the subfamily Danainae (Ackery and Vane-Wright, 1984); it is, moreover, a surprising feature in an aposematic species that is chemically defended (Rothschild *et al*, 1975; Brower *et al*, 1975, 1978; Brown, 1984; JA Edgar *in litt*. to DASS) and supports numerous mimics (Smith, 1973a, 1976, 1979; Owen and Smith, 1993; Owen *et al*, 1994). The conundrum would be resolved, however, if there is a recent history of allopatry, with such populations retaining the ability to interbreed without problems. Therefore, the question we address here is whether, despite hybridisation, a given subspecies – we focus on *alcippus* – is sufficiently isolated from other subspecies to be considered a nascent species.

The formal colour genetics of *D. chrysippus* (Figure 2) is fairly well known (Owen and Chanter, 1968; Clarke *et al*, 1973; Smith 1975a, 1980, 1998; Gordon, 1984; Smith *et al*, 1998). Hindwing colour and pattern are controlled by the autosomal *A* locus: 2a and 2c have the genotype *AA*,

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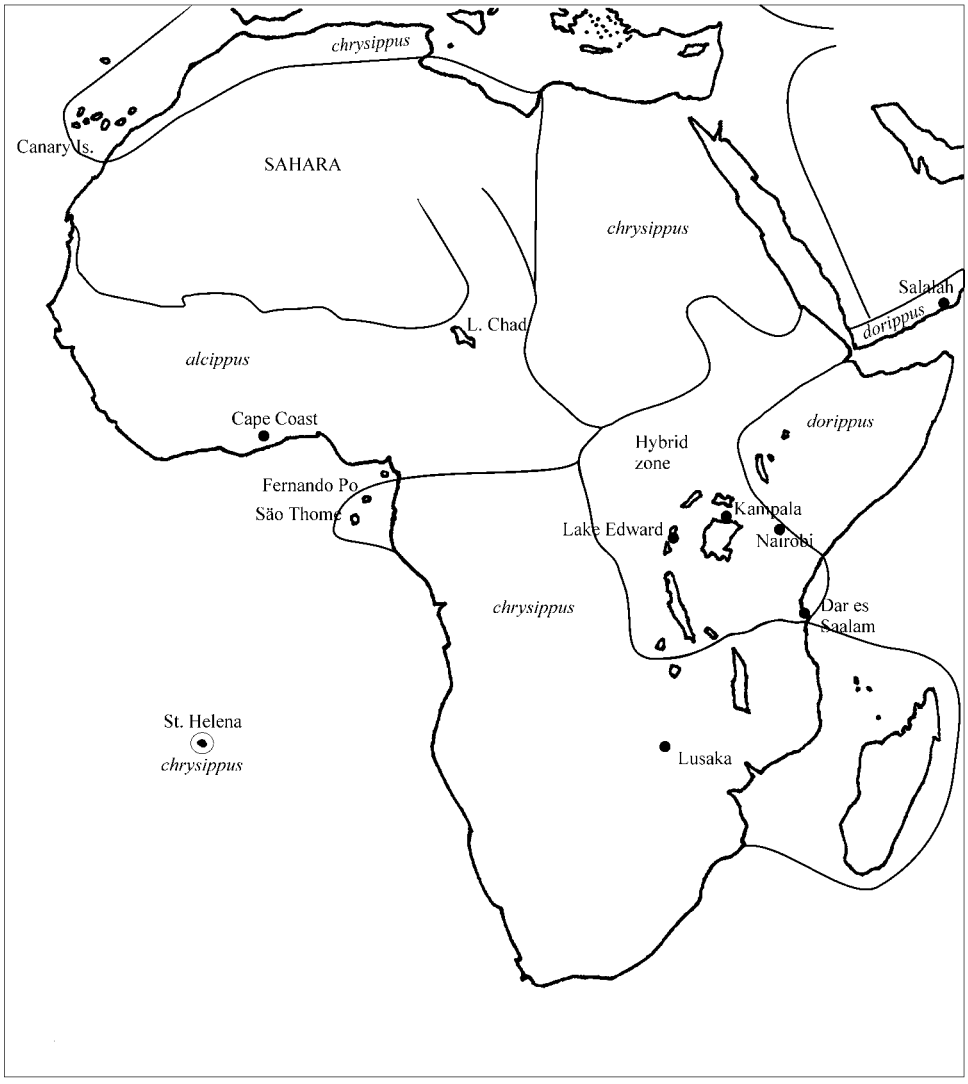

Figure 1 Geographical distribution of the subspecies of *D. chrysippus* in the Afrotropical region, showing the approximate boundaries of the hybrid zone and locations of sampling sites.

whereas 2b and 2d are *aa*. *Aa* heterozygotes are variable being either indistinguishable from *AA* or showing varied amounts of white (weak *alcippus* and weak *albinus*), especially lining the veins, but always less than *aa*. As heterozygotes of intermediate phenotype occur in both sexes, the possibility that the *A* locus might be sex-linked (to either sex chromosome) is ruled out, since females would necessarily be hemizygous for the *A* gene; hence, they would be either *A* or *a* in genotype and, in the case of W linkage, polymorphism would be limited to females.

Ground colour is controlled by the *B* locus with two alleles, brown (*B*) being variably dominant over orange (*b*); *Bb* heterozygotes are either brown or, more usually, show a variable extent of brown on the costal and basal areas of the forewing and the basal area of the hindwing, and the remaining areas are orange. The pattern of the forewing apex is governed by the *C* locus: 2c and 2d are *CC*, whereas 2a and 2b are *cc*. Some *Cc* heterozygotes (*transiens*) are detectable by a row of pale subapical spots on the forewing underside. The *A* locus segregates independently (Clarke *et al*, 1973; Smith, 1975a), whereas the *B* and *C* loci are closely linked with a crossover value of 1.9% in males only (Smith, 1975a).

Despite the ubiquity of hybrids throughout East Africa, three lines of evidence suggest that subspecies of *D. chrysippus* are, albeit imperfectly, sexually isolated (Smith, 1980).

(1) At Dar es Salaam, Tanzania and Cape Coast, Ghana, orange (*bb*) and brown (*B*−) phenotypes mate assortatively (Gordon, 1984; Smith, 1984), as do *chrysippus* (*cc*) and *dorippus* (*C*−) at the former site (Smith, 1975b, 1984). Unfortunately, for the *A* locus we have no direct evidence for assortative mating, although the field data of Owen and Chanter (1968), tested for randomness of mate choice, are suggestive (exact $P = 0.066$, $n = 28$ pairs).

(2) Some, and possibly all, subspecies are independently migratory (Smith and Owen, 1997). Around Nairobi and Dar es Salaam, this behaviour is thought to cause substantial and annually replicated seasonal fluctuation in allele frequencies for the visible *A*–*C* loci

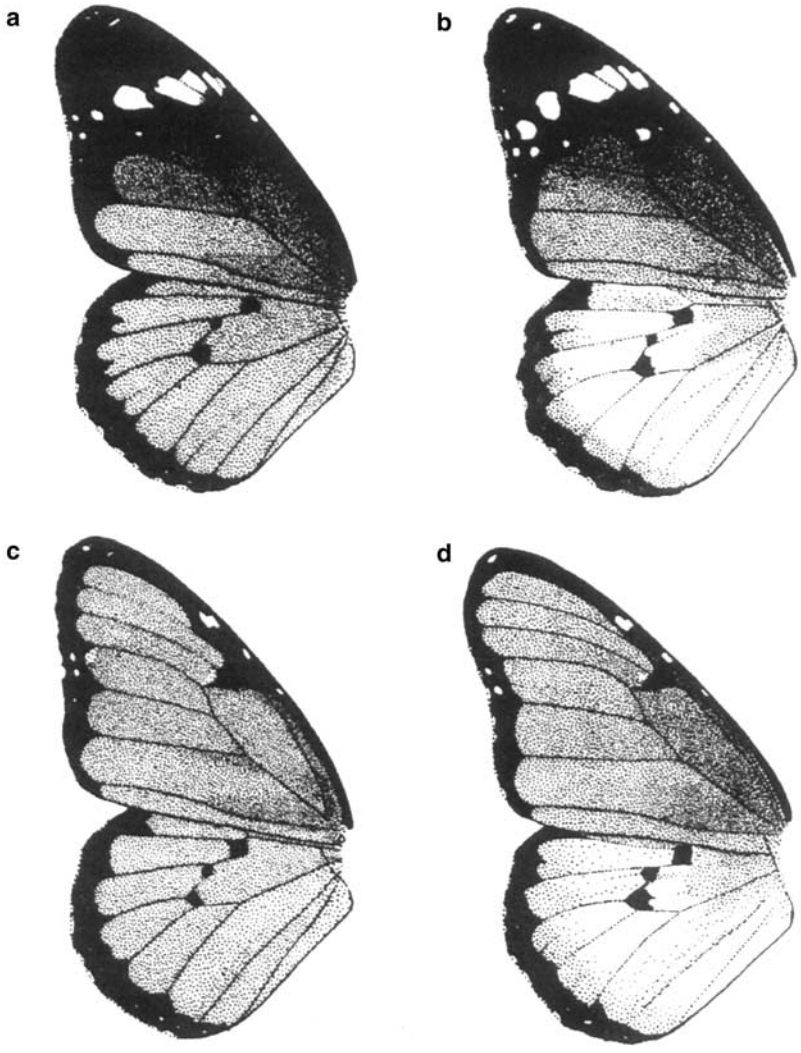

Figure 2 Four major phenotypes of *D. chrysippus* in Africa: (**a**) *chrysippus*, (**b**) *alcippus*, (**c**) *dorippus*, (**d**) *albinus*. The latter is a hybrid phenotype from the cross *alcippus* × *dorippus*. The stippled areas are either tawny orange or nutbrown; the black and white areas are as shown.

(Smith *et al*, 1997). Some sympatry is now a permanent condition in East Africa. However, if substantial numbers of each subspecies are allopatric at some seasons and sympatric at others, with continuous breeding and 12 or so overlapping generations a year (Owen and Chanter, 1968), punctuated sexual isolation must occur in allopatry. Moreover, although gene flow might extend to all populations, those furthest from the hybrid zone may be isolated by distance (Wright, 1969).

(3) Within the hybrid zone at Kampala, Nairobi, Mombasa and Dar es Salaam, but not reported elsewhere, populations are periodically highly female biased (Owen and Chanter, 1968; Smith, 1975c; Gordon, 1984; Smith *et al*, 1998; Jiggins *et al*, 2000; D. Schneider, 1997, *in litt*. to DASS). The cause is an early male-killing *Spiroplasma* bacterium inherited as a cytoplasmic gene and imperfectly transmitted vertically down the female line (Jiggins *et al*, 2000). In Uganda, 40% of females ($n = 90$) were found to be infected, compared to only 4% at Watamu on the eastern coast of Kenya. Estimates of infection frequencies elsewhere in East Africa are obtained from the progenies of wild-caught females; Smith *et al* (1998) reported that 18% ($n = 77$) of females at Dar es Salaam, Tanzania, and 69% ($n = 62$) from Nairobi, Kenya, produced female-biased progenies, predominantly all female broods. On the other hand, small samples of female *D. chrysippus* from Ghana ($n = 6$), South Oman (Salalah) ($n = 15$), South Africa ($n = 4$) and Zambia ($n = 3$) were screened for the presence of *Spiroplasma*; all tested negative. The Oman result is especially interesting as it is the only sample from a polymorphic site to test negative for *Spiroplasma*.

Smith (1975b) found a significantly higher frequency of all-female broods from *chrysippus* compared to *dorippus* females (Figure 2) at Dar es Salaam. Moreover, in field samples, females predominated among *chrysippus*, whereas the SR in *dorippus* was close to 1 : 1. We have drawn attention to heterogeneous SRs within 'morphs' (∼subspecies) throughout the hybrid zone (Smith, 1980; Smith *et al*, 1993, 1997, 1998) and these findings suggest that subspecies may vary in their susceptibility to invasion by *Spiroplasma* and/or unexplored differences in ecology, behaviour or genetics.

In this paper, we present new 12S rRNA mitochondrial gene sequence data and reanalyse *A* locus genotype (nuclear) data from 10 well-separated populations. Thus,

we examine congruence between haplotype and nuclear genotype, within and between subspecies, from allopatric and sympatric ranges, for *prima facie* evidence for sexual isolation. In particular, we investigate concordance, among monomorphic populations and within polymorphic ones, between haplotype and genotype, the latter being the switch between the white hindwinged, western subspecies *alcippus* (*aa*), on the one hand, and (*chrysippus+dorippus*) (*AA*) with orange hindwings, on the other (Figure 2). We review extensive ecological, behavioural and genetic evidence from the hybrid zone, some previously unpublished, that relates to our main hypothesis, namely, that despite much hybridism and long-distance introgression of both cytoplasmic and nuclear genes, there is nevertheless substantial concordance of haplotype and genotype, both within and between populations; this suggests that *alcippus* may be sexually isolated from (*chrysippus+dorippus*), not only in allopatry, but also in sympatry.

Methods

Collection of butterflies

The new data consist of mitochondrial (mt) DNA sequences, 347 bp fragments of the 12S rRNA gene extracted from 10 population samples ($n=169$) of *D. chrysippus*, eight from the Afrotropical region and two from Asia (Table 1). Butterflies were randomly netted in the field, boxed alive and subsequently killed in ethyl ethanoate vapour, immediately before storage in 95% ethanol at -20°C. Wings were removed and retained in papers for reference.

Unpublished field data for two polymorphic populations, in the Cape Verde Islands and South Oman, are presented here for the first time.

Extraction and amplification of DNA

DNA was extracted using a salting-out process (Aljanabi and Martinez, 1997). Thoraces were first briefly dried in a vacuum to remove ethanol and then homogenised using UV-crosslinked plastic homogenisers (Scotlab) in the presence of 300 µl 1 × TEN (250 mM NaCl, 50 mM Tris HCl, 10 mM EDTA, pH 8.0):2% SDS (9 : 1) and 30 µl of 1 mg/ml proteinase-K. Homogenised tissue was left overnight at 37°C (or for 3 h at 55°C), after which 100 µl of 5 M NaCl was added, followed by 15 s vortexing and 10 min high-speed centrifugation. DNA was precipitated from the supernatant with 1 ml of ice-cold absolute ethanol. Samples were centrifuged at high speed for 10 min to produce a DNA pellet. Excess ethanol was decanted off and pellets washed twice with 1 ml of 70% ethanol. After the final wash, all remaining ethanol was removed and the samples air-dried and then resuspended in 50 µl of TE : HPLC H_2O (1 : 4). Aliquots of genomic DNA were further diluted with HPLC H_2O to 25–50 ng/µl for use in polymerase chain reactions. Samples with contaminants that inhibited PCR reactions were divided and one-half was purified in PCR-spin columns (Qiaquick, Qiagen Ltd.), according to the manufacturer's recommendations. This procedure yielded ample DNA for PCR.

The region from the 12S rRNA gene was amplified using primers based on conserved sequences within sites (cf. Simon *et al*, 1994; Loxdale and Lushai, 1998). Forward (F) and reverse (R) primers were: 12S rRNA-F, 5′-aagagcgacgggcgatgtgt-3′ and 12S rRNA-R, 5′-aaactaggattagataccctattat-3′. For PCR reactions, DNA templates were aliquoted in a PCR-reaction mix [0.1v of 10 × *Taq*-polymerase reaction buffer (Appligene-Oncor), 0.2 mM/µl dNTPs (Pharmacia), 10 pmol primer, 2.0 U *Taq* DNA polymerase (Appligene-Oncor) in a total volume of 25 µl and overlaid with mineral oil]. PCR amplifications were carried out in an Omnigene (Hybaid Ltd.), using a hot start at 94°C/2 min (1-cycle), denaturation at 92°C/1 min, annealing at 45–58°C/1 min (dependent on primer specificity), extension at 72°C/2 min (30-cycles) and a final extension step of 72°C/7 min (1-cycle). Amplified PCR products were visualized on a 1% TBE agarose gel and excised gel fragments were purified using gel extraction columns (Qiagen).

Sequencing of DNA

One template sample from *D. chrysippus* from Malaysia (50 ng/µl concentrations) was autosequenced for the 12S region (Oswell Ltd) in both directions. The sequence was then used as a control for further sequencing. Aliquots of purified PCR fragments were tested on agarose gels for concentration and 50–100 ng of the remaining sample was used in asymmetric reactions with the diluted aliquots of the 'forward primer' (0.2 pmol). Approximately half the reactions were sequenced manually with a cycle sequencing kit (Pharmacia Biotech) and ^{35}S, adopting the dideoxychain-termination procedure (Sanger *et al*, 1977). These were then visualised by autoradiography. The remaining reactions were carried out in-house using ABI-Big-Dye® terminator cycle sequencing (Perkin-Elmer).

Both sense and antisense fragments were sequenced. Manual sequences were read into text files by eye and autosequence files were screened by eye using CHROMAS 1.45, exported as text files and formatted as interleaved sequences for multiple alignment by CLUSTAL × (1.5b) (Thompson *et al*, 1994). Sequences were also preliminarily screened against GenBank to compare them for sequence homology against known sequences in the BLAST-NR database (eg 98–92% homology was shown with 12S rRNA sites in *Eurema nicippe* and *Tortricodes alternella* (Lepidoptera)).

Results

12S rRNA haplotypes and their biogeography

Of the 10 populations sampled, five were polymorphic for both haplotype and genotype, and five monomorphic for both (Table 1). All butterflies were readily assigned to either of the two widespread haplotypes, ST (standard) and GH (Ghana), distinguished by a TA/– indel at sites 199–200 (Table 2). The five populations that were monomorphic for colour genes were also invariant for haplotype but 16 minor ST and GH variant haplotypes ($\leqslant$2 bp differences, $n=19$ individuals) were found in polymorphic populations (Table 2). Although our samples give low geographical coverage for such a vast study area, the existing data suggest that GH has a western distribution in Africa, whereas ST predominates over the remainder of the continent and extends eastwards across Asia at least as far as Malaysia.

Table 1 Samples of *D. chrysippus* scored for *A* locus genotype and sequenced for the 12S rRNA mitochondrial gene

Provenance	*Subspecies*	A *locus genotypes*	*12S haplotypes*		
			ST	*GH*	N
Monomorphic sites					
Cape Coast, Ghana	*alcippus*	*aa*	0	15	15
Lusaka, Zambia	*chrysippus*	*AA*	8	0	8
North Oman	*chrysippus*	*AA*	5	0	5
Petiala, India	*chrysippus*	*AA*	4	0	4
Penang, Malaysia	*chrysippus*	*aa*	13	0	13
Polymorphic sites					
Kampala, Uganda	*chrysippus, alcippus, dorippus*	*AA, Aa, aa*	12	18	30
Athi River, Kenya	*chrysippus, alcippus, dorippus*	*AA, Aa, aa*	15	10	25
Masai Mara, Kenya	*chrysippus, alcippus, dorippus*	*AA, Aa, aa*	4	2	6
Dar es Salaam, Tanzania	*chrysippus, alcippus, dorippus*	*AA, Aa, aa*	33	14	47
South Oman (Dhofar)	*chrysippus, alcippus, dorippus*	*AA, Aa, aa*	9	7	16

Notation: ST, standard; GH, Ghana.

Table 2 Variable sites for the 347 bp 12S rRNA sequences included in this paper

Provenance of samples	*Haplotype*	*00111112222222220436889024777889 7529169008089492*	N
Malaysia, India, Zambia, Oman, Kenya, Uganda, Tanzania	ST1	CT-AAATATCATATAT	93
Athi River, Kenya	ST2	.A.	1
Athi River, Kenya	ST3	.G.	1
Athi River, Kenya	ST4	.AT.	1
Masai Mara, Kenya	ST5	A.	1
Kampala, Uganda	ST6	. .TT	1
Kampala, Uganda	ST7	. . .T	1
Dar es Salaam, Tanzania	ST8	. . . . . TT.	2
Dar es Salaam, Tanzania	ST9	. . . . . A.	1
Salalah, Oman (Dhofar)	ST10	. . . . . --. . .	1
Total			103
Ghana, Uganda, Kenya, Tanzania, Oman	GH1	. . . . .--.	57
Athi River, Kenya	GH2	. . . . .---.	1
Masai Mara, Kenya	GH3	. . . . C.--.	1
Kampala, Uganda	GH4	. . . . C.--. . . . .-. .	1
Kampala, Uganda	GH5	. . . . .T--.	1
Dar es Salaam, Tanzania	GH6	. . . . . --. . . . . A	1
Dar es Salaam, Tanzania	GH7	. . . . . --. . . . . T.	3
Salalah, Oman (Dhofar)	GH8	A. . . . . --.	1
Total			66

GenBank accession numbers for representative sequences: AF394676-82.

Haplotype and sex

Within polymorphic populations, the distribution of haplotypes in lumped data differs significantly between the sexes, GH being predominant (72.5%) in males and ST (71.4%) in females ($\chi^2_1 = 5.28$, $P < 0.02$). Although the data are insufficient to show that each sample is individually deviant, the trend lies in the same direction in all five hybrid zone populations ($P = 0.03$, one-tailed). It could be tentatively suggested that ST (*chrysippus+dorippus*) butterflies are more likely than GH (*alcippus*) to be infected with male-killing *Spiroplasma*, although a larger and more extensive sampling programme would be needed to predict its epidemiology within the hybrid zone.

Penetrance of the a allele in Aa heterozygotes

To establish genotype and allele frequencies at the *A* locus, without which randomness of mating, Hardy–Weinberg equilibria (HWE) and concordance between cytoplasmic and nuclear genes cannot be estimated, we needed a better estimate of penetrance of the *a* allele in *Aa* heterozygotes than was achieved by Smith (1998). An experienced breeder is readily able to distinguish the heterozygous *Aa* phenotype from *aa*, but not from *AA*: this is because the *Aa* phenotype ranges from butterflies with a small white patch on the hindwing, through individuals with a scattering of white scales, especially along the margins of veins, to those from which white is absent. Accordingly, we have re-examined the *Aa*

offspring ($n=294$, Table 3) from $AA \times aa$ and $Aa \times aa$ progenies ($n=13$) from Kenya and Tanzania (Smith *et al*, 1998); the *a* allele was scored as penetrant if *any* white scales were detectable in the relevant area of the hindwing, viewed through a $\times 2$ hand lens. Only progenies obtained from sympatric parents were used because penetrance of *a* in heterozygotes may differ if the parents are from geographically distant sources. In practice, of course, this distinction may not be strictly sustainable if populations are mobile, with some *AA* and *aa* butterflies being cyclically allopatric and sympatric at different seasons, as suggested for *CC* and *cc* butterflies by Smith and Owen (1997).

Penetrance of the *a* allele in *Aa* heterozygotes (Table 4) differs very significantly ($P<0.0001$) between the sexes. For males penetrance is 0.654 (95% CI, 0.570–0.738), whereas in females it is only 0.335 (95% CI, 0.261–0.409). There is no epistatic interaction from either *B* or *C* locus that affects this trait. The highly significant interaction between *C* genotype and sex is a sampling effect, reflecting the predominance of *cc* among females (71.1%) and *C-* in males (68.5%), the majority of the former emanating from all-female broods (Smith *et al*, 1998). The two marginally significant *B* locus interactions, with the *C* locus and sex, are explained by tight linkage of the *B* and *C* loci (Smith, 1975a). The significant second- and third-order interactions are not readily explained.

Table 3 $2\times2\times2\times2$ contingency table for *Aa* offspring from 13 $AA \times aa$ and $Aa \times aa$ progenies raised from wild-caught pairs at Dar es Salaam (*n*=9) and Nairobi (*n*=4) (Smith *et al*, 1998), classified by four criteria, penetrance of the *a* allele, *B* locus genotype (*B−/bb*), *C* locus genotype (*C−/cc*) and sex

		C−		Cc		*Total*
		♂	♀	♂	♀	
a penetrant	*B−*	18	3	23	13	57
	bb	41	15	3	24	83
a impenetrant	*B−*	11	20	11	20	62
	bb	19	25	4	44	92
Total		89	63	41	101	294

Table 4 Analysis of χ^2 for the data in Table 3

Effect tested	χ^2	*d.f.*
Penetrance of *a* allele in *Aa* genotype × *B* genotype (*B−/bb*)	0.006	1
Penetrance of *a* allele in *Aa* genotype × *C* genotype (*C−/cc*)	1.165	1
Penetrance of *a* allele in *Aa* genotype × sex	29.488***	1
B genotype (*B−/bb*) × *C* genotype (*C−/cc*)	5.128*	1
B genotype (*B−/bb*) × sex	6.168*	1
C genotype (*C−/cc*) × sex	26.218***	1
Second-order interaction at *A* locus	0.222	1
Second-order interaction at *B* locus	10.667**	1
Second-order interaction at *C* locus	0.000	1
Second-order interaction for sex	3.932*	1
Third-order interaction	10.829**	1
Total	93.823***	11

*$P<0.05$; **$P<0.01$; ***$P<0.001$. Partition of χ^2 follows the method of Lancaster (1951) and Lewis (1962).

Concordance between A locus genotype and haplotype is maintained in sympatry

Concordance of haplotype and *A* genotype, ST with *AA* and GH with *aa*, for the vicariant and monomorphic populations in Africa, Arabia and India is 100% (exact $P=1.0\times10^{-9}$) (Table 1). Here we should point out that the exception appears to be *D. chrysippus* in Malaysia, where, except in the northern border province of Kedah, form *alcippoides* is monomorphic for white hindwing (Corbet *et al*, 1992; DASS, unpublished) and yet our sample is invariable for ST. As the West African and Malaysian populations are highly disjunct, the recessive *a* allele may be symplesiomorphic; alternatively, the same mutation may have occurred twice independently. On the other hand, although rare *Aa* hybrids from Kedah resemble those from Africa (DASS, unpublished), confirmation that white hindwing in Malaysia is controlled by the *A* locus is lacking.

The degree of haplotype–genotype concordance in polymorphic samples from the Afrotropics is set out in Table 5, where the data are presented both uncorrected and corrected for penetrance of the *a* allele in the *Aa* genotype in males and females. The association of ST with *A-* and of GH with *aa* is highly significant in both cases. For corrected data, $\chi^2_2=39.2$, $P<0.0001$, whereas for uncorrected data, $\chi^2_2=14.6$, $P<0.001$. Similar tests for association of ST with the *A* allele and of GH with *a* are, respectively, for uncorrected and corrected data, $\chi^2_1=21.9$, $P<0.001$ and $\chi^2_1=43.4$, $P<0.0001$. As the *a* allele has low frequency in most hybrid zone samples, tests on individual samples show a significant association for haplotype and allele frequencies only at Kampala ($\chi^2_1=8.25$, $P<0.01$); however, all five hybrid zone samples (Table 1) deviate from randomness in the same direction ($P=0.03$, one-tailed).

The haplotype–*A* genotype concordance of GH/*aa* (*alcippus*), compared to ST/*AA* (*chrysippus+dorippus*), even in conservative tests using data uncorrected for *Aa* genotype frequency, strongly indicates that the subspecies have incomplete sexual isolation in sympatry. Were it otherwise, populations such as these, infected with bacterial symbionts that are inefficiently transmitted vertically, are expected rapidly to lose mtDNA diversity through constant movement of mtDNA variants from infected to uninfected hosts (Hurst *et al*, 1997). If, however, phenotypes were partially isolated by mate choice, such loss of mtDNA diversity would be forestalled.

Polymorphic populations that have normal sex ratios

We have new field data, for two polymorphic *D. chrysippus* populations, both of them far distant from East Africa, that have normal (1:1) sex ratios and are, therefore, by deduction probably free from *Spiroplasma* infection.

On the island of Saõ Vicente, Cape Verde Islands, the *D. chrysippus* population, sampled in October 2000, is monomorphic (*bbcc*) at the *B* and *C* colour gene loci and polymorphic only for the *A* gene. The *A* locus poly-

Table 5 Frequencies of 12S haplotype, *A* locus genotype and *A* locus alleles in polymorphic (hybrid zone) samples of *D. chrysippus* (Frequencies in parentheses are corrected for penetrance of the *a* allele in the *Aa* genotype.)

12S Haplotype	A *locus genotypes*				A *alleles*		
	A–	Aa	aa	N	A	a	N
ST	63 (63)	7 (7)	3	73	134 (130)	12 (16)	146
GH	29 (16)	11 (24)	11	51	69 (65)	33 (37)	102
Total	92 (79)	18 (31)	14	124	203 (195)	45 (53)	248

Table 6 Frequencies of *C* locus phenotypes in a field sample of *D. chrysippus* from Salalah, South Oman, 21–23 October 1998. Figures in parentheses are estimated genotype frequencies after correction for penetrance (Smith, 1998)

	C *locus phenotypes*			
	C–	Cc	cc	N
Males	34	16	10	60
Females	18	6	6	30
Total	52 (25)	22 (49)	16 (16)	90

morphism is unexpected and may be recent, since all museum specimens from the Cape Verde Is. we have examined ($n = 23$) are monomorphic for *aa* (*alcippus*). Since butterflies on the wing were highly dispersed and difficult to catch, the population was sampled by rearing adults from eggs collected off the foodplant, *Calotropis procera*. Of 27 adults successfully reared, 15 were female (seven *Aa*, eight *aa*) and 12 male (three *Aa*, nine *aa*): thus, there is neither evidence for a female-biased SR ($\chi^2_1 = 0.33$, NS), nor for allele frequencies that differ between sexes ($\chi^2_1 = 0.44$, NS) or heterozygote excess (HE). Indeed, the limited data do not reject a null hypothesis that the population is in HWE ($\chi^2_1 = 0.15$, NS). The absence of *Spiroplasma* is inferred from the 1 : 1 SR.

More extensive field data were collected for the population at Salalah, South Oman (Table 1) that is monomorphic for *bb*, but polymorphic for both *A* and *C* genes. Here, the *a* allele has a low frequency (0.028) but, since widespread HE has been found for the *C* locus throughout East African hybrid populations (Smith *et al*, 1997), the *C* polymorphism at Salalah (Table 6) can be used to test the hypothesis that HE is linked to *Spiroplasma* infection.

As females ($n = 15$) from this population tested negative for *Spiroplasma* (Jiggins *et al*, 2000) and the SR for the field sample is 2 : 1, there are neither grounds to expect a female-biased SR, nor any evidence for it. Furthermore, the frequency distribution of phenotypes between the sexes is not heterogeneous ($\chi^2_2 = 0.53$, NS), nor do the data, corrected for penetrance of the *c* allele in the *bbCc* genotype (Smith, 1998), reject an hypothesis that the population is in HWE at both *C* locus ($\chi^2_1 = 1.40$, NS) and *A* locus (Table 9). An important caveat here, however, is that the penetrance estimate for *c* based on East African material may not be valid for this far-distant population.

Discussion

We attempt to address two questions. (1) Is the genetic, behavioural and biogeographical evidence relating to subspecies of *D. chrysippus*, sufficient to suggest that *alcippus*, in particular, is sexually isolated from other subspecies of the complex and is, thus, a biological species? (2) Is the paradoxical existence of widespread hybridism among subspecies in East Africa a fatal *objection* to a specific status for *alcippus* under the biological species concept or, alternatively, is it a maladaptive consequence of the differential invasion of subspecies by male-killing *Spiroplasma* bacteria that cause severely female-biased SRs and enforce heterotypic mating by infected females?

Sex and A locus allele frequencies

The samples from Athi River (Tables 7 and 8) suggest that *A* locus allele frequencies differ significantly between the sexes. Although 'spot' samples at Athi River are variable between months and years (Table 9), all the deviations describe the same trait, the *a* allele invariably exceeding expectation in females compared to males ($P = 0.016$). The unique sample from Lake Edward (for which we have no haplotype data) is, in this respect, similar to the pooled samples from Athi River. On the other hand, in the Kampala and Dar es Salaam populations, it is the *A* allele that has a significantly higher than expected frequency in females (Table 7). We may deduce from these data that substantial sex differences for allele frequencies at the *A* locus are a general feature of all these hybrid zone populations, as previously established for the *B* and *C* loci (see Table 8 in Smith *et al*, 1997). Therefore, as the sexes differ in genetic profile, mate selection must be canalized towards heterotypic pairing. This is especially the case at times when the SR is strongly biased to females, since rare males will overwhelmingly encounter heterotypic females that are denied choice. Thus, as countervailing prezygotic isolation does not prevent eventual copulation, HE is inevitable. HE in three highly female-biased populations (Table 8), two from Athi River and one from Kampala, is effectively confined to females that must be obliged to mate with scarce males, predominantly of different genotype from themselves and from bisexual broods. In a small sample, this scenario was undoubtedly misinterpreted as disassortative mating in a highly

Table 7 *A* locus allele counts (expected numbers in parentheses) in males and females from four polymorphic populations of *D. chrysippus*. Classes that exceed expectation are underlined

Population	*Males*		*Females*		N	χ^2_1
	A	a	A	a		
Athi River	404 (382)	96 (118)	846 (868)	290 (268)	1636	7.71**
Lake Edward	66 (57)	56 (65)	24 (33)	48 (39)	194	7.85**
Kampala	52 (62)	162 (152)	71 (61)	137 (147)	422	4.94*
Dar es Salaam	4381 (4422)	367 (326)	2921 (2856)	145 (201)	7814	27.39***

Table 8 Frequencies of *A* locus genotypes in each sex of *D. chrysippus* observed# (expected) in three populations where there is heterozygote excess

Sample	*Sex*	*Observed*[a] *(expected) genotypes*			N	χ^2_1
		AA	Aa	aa		
Athi River, 01.86	♂	2 (2.4)	3 (2.1)	0 (0.5)	5	—
	♀	0 (10.2)	**46 (25.7)**	6 (16.2)	52	32.72***
Athi River, 02.89	♂	12 (13.2)	8 (6.1)	0 (0.7)	20	—
	♀	44 (51.8)	**69 (53.4)**	6 (13.8)	119	10.11**
Kampala, 12.91	♂	4 (6.4)	44 (39.6)	59 (61.0)	107	1.03 ns
	♀	0 (12.1)	**71 (46.7)**	33 (45.2)	104	21.69***

Notation: not significant; $^{**}P<0.01$; $^{***}P<0.001$.
[a]Observed numbers are corrected for penetrance of the *a* allele in *Aa* heterozygotes.

Table 9 Goodness-of-fit to Hardy–Weinberg equilibria (H_0) of *A* locus genotypes in polymorphic populations of *D. chrysippus*

Site	*Date*	*Stage collected*	*Frequency* (q) *of* a *allele*	*Fit of observed genotype frequencies*[a] *to HWE*				
				AA	Aa	aa	N	χ^2_1
Athi River	01.86	Egg	0.535	2	**49**	6	57	32.00***
Athi River	01.94/95	Adult	0.107	88	20	2	110	0.02 NS
Athi River	02.89	Egg	0.372	56	77	6	139	30.49***
Athi River	04.94	Adult	0.187	144	74	5	223	1.85 NS
Athi River	05.87	Egg	0.224	106	53	12	171	2.30 NS
Athi River	07–08.87	Egg	0.309	19	14	5	38	0.14 NS
Athi River	07.86	Egg	0.444	4	12	2	18	2.57 NS
Athi River	11.86	Egg	0.089	52	9	1	62	0.03 NS
Kampala	12.91	Adult	0.707	4	**115**	92	211	21.69***
Lake Edward	09.93	Adult	0.536	21	48	**28**	97	12.34**
Dar es Salaam	01.74–12.75	Adult	0.067	3450	*402*	**55**	3907	92.88***
Salalah	10.98	Adult	0.028	86	3	1	90	0.20 NS

Notation: as Table 8.
[a]Observed frequencies are corrected for penetrance of the *a* allele in the *Aa* genotype. Genotype numbers in bold and italic type are, respectively, significantly above or below HWE expectation.

female-biased population at Dar es Salaam (Smith, 1973b).

Hardy–Weinberg equilibria (HWE)

Samples from wild populations (Smith *et al*, 1997; Table 9) were collected in a number of different ways, each method having its merits and disadvantages for estimating allele frequency, SR and HWE. The majority of Athi River samples consisted of eggs. Most were collected in a single day (with the exception of July–August 1987) and subsequently raised to adults in the laboratory. These samples give the least biased estimates of sex and morph ratios. Samples of flying adults have the disadvantage that the more active males often predominate, even when the population is known to be female biased (eg Kampala, Owen and Chanter, 1968; Dar es Salaam, Smith, 1975b, 1980). In theory, samples could also be biased in other ways such as subspecies differences in migration routines (Smith and Owen, 1997), variation in daily rhythms or microhabitat preferences, although the two latter factors have not yet been investigated. Samples accumulated over long periods, for example, at Dar es Salaam, could in theory show a spurious excess or deficit of heterozygotes resulting from pooling heterogeneous data (Wahlund, 1928). However, as the frequency of the *a* allele at Dar es Salaam (1972–75) was invariably low

($q = 0.067$, 95% CI = 0.061–0.073, $n = 6570$), and not seasonally variable (Smith *et al*, 1997), a Wahlund effect can probably be ruled out.

As *D. chrysippus* is both migratory and noted for non-random mating, HWE is not to be expected. Although HWE at the *A* locus may be obtained at Athi River (Table 9), sample sizes are generally inadequate and two of them, January 1986 and February 1989 (Table 8), show that highly significant HE occurs periodically. This phenomenon was also found for the *C* locus in all Athi River samples except that for January 1986 (Smith *et al*, 1997). Similar HE at the *A* locus is apparent in the Kampala sample that was collected over a period of 2 weeks (Smith *et al*, 1993). These data show that in heavily female-biased populations (Owen and Chanter, 1968; Smith *et al*, 1993, 1997, 1998; Jiggins *et al*, 2000), the excess of *A* locus heterozygotes may be confined to females (Table 8) that must be predominantly the offspring of mothers infected with *Spiroplasma*. Thus, if hybridism is effectively confined to one sex, HE is an evolutionary *cul-de-sac* that may impede but will not prevent the evolution of sexual isolation. Indeed, selection should favour chauvinistic males whose preference is for homotypic females that are less likely to carry *Spiroplasma*. Theoretically, if males are able to detect and mate preferentially with uninfected females, the advantage to the symbiont of infecting more females will be balanced, both by the delay to its host attracting a mate and, possibly, reduced host fecundity (see below).

The long-term work at Dar es Salaam (1974–1975), where *A* locus gene frequencies are stable, reveals a highly significant heterozygote deficit that suggests either assortative mating or relative heterozygote unfitness. Either outcome supports sexual isolation. The Lake Edward sample, collected in a single day, has an excess of *aa* and a deficiency of *AA* that could result either from recent immigration of the former or emigration of the latter.

Dynamics of the East African hybrid zone

It is now established that the following coincident phenomena are generally encountered in populations of *D chrysippus* in the East African hybrid zone (Smith *et al*, 1997, 1998 and references therein): (1) populations are generally heavily female biased; (2) haplotype (this paper) and nuclear gene frequencies differ very significantly between the sexes; (3) heterozygotes may be either in excess, especially among females, or in deficit, and HWE is atypical; (4) 'morphs' differ in migratory behaviour; (5) allele frequencies are subject to marked seasonal variation and (6) paradoxically, although empirical evidence for mate choice suggests it is normally assortative, heterotypic pairing frequently predominates in practice. Applying Occam's razor, we suggest that the co-occurrence throughout an area larger than Western Europe, of six phenomena otherwise unknown in *D. chrysippus*, most likely share a single explanation.

Whereas previous work has concentrated on the linked *B* and *C* loci, we now show that the above phenomena also embrace the unlinked *A* locus. If future experimental work should confirm that subspecies differ in their susceptibility to invasion by *Spiroplasa*, differing gene and haplotype frequencies between the sexes in mixed populations would be readily understood. The surfeit of females in an infected subspecies would be forced to mate with males of an uninfected one that has a relatively high SR. As the bacterial gene is passed only down the female line, the preponderance of heterozygotes among females would thus be explained. Furthermore, the associated haplotype of these females would inevitably hitch-hike with the bacterium (Hurst *et al*, 1997) and the W chromosome (Jiggins *et al*, 2000). That this has in fact occurred is suggested by the finding that the ST (*chrysippus+dorippus*) haplotype has a significantly higher frequency (71.4%) in hybrid zone females, whereas GH predominates (72.5%) in males. As we have never found a wild virgin female, we assume all females, whether infected or not, are eventually mated. However, when males are scarce, they should mate preferentially with uninfected females to produce sons. Furthermore, if uninfected females acquire more spermatophores than infected ones, and the additional nutrient resources are used for egg production, as in *D. plexippus* (Wells *et al*, 1993; Zalucki, 1993), males that choose such females could be advantaged by higher fecundity, balanced by greater exposure to shared paternity. *D. gilippus* females may acquire up to 15 spermatophores (Pliske, 1973) and the closely related *D. chrysippus* is similarly promiscuous (Smith, 1984). Thus, male preference for uninfected over infected females could be tested by spermatophore counts using material typed for *Spiroplasma* infection.

Our data indicate that, at Kampala and Dar es Salaam, males could in theory preferentially sire bisexual progenies by choosing females that carry a penetrant *a* allele as this advertises their lower probability of being infected. Similar data suggest that the *b* and *C* alleles may also serve, in these and other populations, as advertisements for females less likely to be infected (Smith *et al*, 1997, 1998). Hence, if homotypic males tend preferentially to choose such females, they will mate assortatively. However, in populations overwhelmingly infected with *Spiroplasma*, as is the case seasonally at Athi River (Smith *et al*, 1997), SRs are very low and females will be best served by mating with any available male. In such an event, the majority of pairings will be heterotypic as female choice would be severely limited. The mating behaviour of *D. chrysippus* in the hybrid zone would clearly reward further study.

Polymorphic populations outside East Africa

The hypothesis that rampant hybridism in East Africa is an enforced consequence of *alcippus* and (*chrysippus+dorippus*) being unequally susceptible to *Spiroplasma* infection has implications for the two populations outside East Africa that are polymorphic at the *A* locus and probably free from *Spiroplasma* infection. Sex ratios are predicted to be normal (1:1), mating random, or possibly, by analogy with the *B* and *C* loci, assortative, and heterozygote excess absent; furthermore, allele frequencies for an autosomal gene such as *A* are expected to be equal across the two sexes. In the Cape Verde Islands and Oman, where *Spiroplasma* is reasonably inferred (from negative tests and/or a 1:1 SR) to be absent, the population parameters we have measured apparently answer these expectations in all respects, although the sample size in the former case is small. Our hypothesis is, therefore, strengthened by finding that

these two fringe populations, from which the bacterial symbiont is probably absent, are free from all the bizarre phenomena that are normally encountered in East African polymorphic populations.

Conclusions

Concordance of haplotype and genotype in the partially vicariant subspecies ST/*AA* (*chrysippus+dorippus*) and GH/*aa* (*alcippus*), which is imperfectly maintained in the face of extensive hybridism where they meet, suggests that sexual isolation sufficiently robust to withstand periodic introgression has evolved. Mating behaviour at the *A* locus has not been studied but, as we have good evidence for assortative mating at the *B* and *C* loci, is expected. Furthermore, a deficiency of *A* locus heterozygotes in the 2-year study at Dar es Salaam also supports sexual isolation. The pervasive, but seasonally variable, male-killing *Spiroplasma* infections, which on present evidence are confined to the East African hybrid zone (Jiggins *et al*, 2000), are the only known cause of the low SRs and they probably rarely invade all subspecies equally. Therefore, the consequent asymmetry of mate choice, far from being the result of hybridism as suggested by Smith *et al* (1998), may be its principal causation. On this interpretation, hybridism and HE are maladaptive and arise as disadvantageous side effects of a severely skewed SR. The concordance in *alcippus* of a diagnostic haplotype and genotype, together with a substantially vicariant distribution, suggest that, despite hybridisation with *chrysippus* and *dorippus* where their ranges overlap, it should be treated as a separate species.

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