Selective increase of a rare haplotype in a land snail hybrid zone

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In hybrid zones, which are regions where genetically differentiated populations of organisms meet and produce hybrids, allozyme studies have often revealed unexpected alleles. The cause of this 'hybrizyme' or 'rare allele' phenomenon has been elusive, although it has been variously ascribed to natural selection or increased mutation rates. If the latter hypothesis is correct, selectively neutral markers should demonstrate increased variability in contrast to expressed markers such as allozymes. In this study, we screened selectively neutral variation in an intron of the calmodulin (*CaM*) gene in a hybrid zone between two subspecies of the Greek land snail *Albinaria hippolyti*. In previous allozyme studies, this hybrid zone has been shown to exhibit the rare allele phenomenon. We used a variant of the single-strand conformational polymorphism technique to detect seven haplotypes in both parental taxa. In the zone, one of these occurs at unexpectedly high frequencies. Since no additional mutants were found, we concluded that this is the result of selection.

Keywords: rare allele; hybrid zone; *Albinaria hippolyti*; speciation genes; SSCP

1. INTRODUCTION

Hybrid zones are narrow regions where members of genetically distinct populations meet, mate and produce hybrids (Barton & Hewitt 1985). They have been successfully exploited as 'natural laboratories' (Hewitt 1988) for studying the ecology and genetics of species differences in organisms as diverse as firebelly toads (Szymura & Barton 1986, 1991), land snails (Woodruff & Gould 1987; Chiba 1993), grasshoppers (Hewitt 1993) and irises (Arnold *et al.* 1990). In many hybrid zones, certain allozymes have been observed at high frequencies, whereas the same allozymes are rare or absent in the parental taxa. This effect has been referred to as the 'rare allele' (Sage & Selander 1979) or 'hybrizyme' (Woodruff 1989) phenomenon (we will use these terms interchangeably) and it is surprisingly common. Barton & Hewitt (1985) reviewed 23 studies that included thorough electrophoretic data, 19 of which showed the phenomenon. Since then, several additional examples have been published (Keenan 1994; Schilthuizen 1995; Guiller *et al.* 1996). The effect can sometimes be pronounced: in a hybrid zone between the Bahamian land snails *Cerion stvensoni* and *Cerion fernandina*, an *Mdh-1* allele was found at frequencies of up to 0.26, while it was undetectable outside the zone (Woodruff 1989) and in the hybrid zone between subspecies of the deer mouse *Peromyscus californicus*, Smith (1979) found alleles at the *Me-1* and *Pgm-1* loci that were ten to 20 times more common than in non-hybrid populations.

DNA sequencing studies (Bradley *et al.* 1993; Hoffman & Brown 1996) have indicated that hybrizymes are not the result of transposition or recombination between

parental alleles, as had been suggested previously (Watt 1972; Golding & Strobeck 1983; Woodruff 1989). Instead, they are related to other alleles by single nucleotide substitutions. Consequently, two proposed explanations for the rare allele phenomenon remain: (i) selective advantage of otherwise slightly deleterious alleles (Barton & Hewitt 1985), or (ii) increased rates of nucleotide substitution in hybrids (Thompson & Woodruff 1978; Barton *et al.* 1983; Barton & Hewitt 1985; Woodruff 1989; Schilthuizen & Gittenberger 1994). The latter has been the most popular, but both suffer from problems. It is hard to see how alleles which are normally rare and presumably mildly deleterious should be positively selected for in the hybrid zone. This appears to weaken the selection hypothesis. The mutation hypothesis has the drawback that usually a single allele at a locus reaches high frequencies. If substitution rates in hybrids were elevated, one would expect numerous new variants, although evolutionary constraints have been postulated to account for this (Schilthuizen & Gittenberger 1994).

In this study, we investigated the rare allele phenomenon in a hybrid zone between the Greek land snails *Albinaria hippolyti holtzi* and *Albinaria hippolyti aphrodite*. These snails have generation times of at least three years and are exceedingly difficult to culture in the laboratory (Schilthuizen 1994), so hybridization can only be studied in the field. Previous work (Schilthuizen 1995) has revealed that the hybrid zone is not associated with any obvious ecotone, and steep coincident clines (80–260 m wide) are present in numerous conchological, anatomical and allozyme characteristics. The clines are probably maintained by a balance between dispersal and selection against hybrid genotypes. Its age is unknown, but mitochondrial DNA divergence points to a common ancestor

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Figure 1. Map of the study area 5 km west of the town of Kroussónas, central Crete. Contours are given at 50 m altitude intervals. The thick shaded line indicates the approximate width and position of the morphological hybrid zone between *A. h. aphrodite* (to the north) and *A. h. holtzi* (to the south). The interrupted line is a path. Letters denote sample sites (explained in §2(a)).

for the two subspecies several million years ago (Douris *et al.* 1998; D. Thomaz, unpublished data).

A hybrid allele at the *sAat* locus has been demonstrated previously in the hybrid zone, reaching frequencies of up to 0.7 in the centre while absent or rare elsewhere (Schilthuizen & Gittenberger 1994). In the present study, we focus on a putative neutral sequence (an intron in the calmodulin (*CaM*) gene), because this may allow a distinction between the mutation and selection hypotheses. Since molecular evolution in this marker should be unconstrained, any increased mutation rates should result in the presence of many new sequence variants (haplotypes). Alternatively, if hybrid alleles are maintained by selection, a neutral marker should either show no anomalies or (in the case of linkage with a selected gene) a pattern similar to that seen in hybrid alleles.

2. MATERIAL AND METHODS

(a) *Snails*

Live adult snails were collected in November 1997 near the town of Kroussónas in the mountains of central Crete. Four sample sites were located 1–2 km away on either side of the morphologically determined hybrid zone and belonged to typical *A. h. holtzi* (samples HE and HW) and *A. h. aphrodite* (samples AE and AW) populations. The remaining two samples (CE and CW) were taken from the centre of the hybrid zone (figure 1). Each of the six samples was taken in an area of 10 m × 50 m. In addition, a sample of *Albinaria hippolyti harmonia* was used for comparison. This was collected near Tilisos (Crete) in September 1992. Voucher specimens have been deposited in the collection of the National Museum of Natural History 'Naturalis', Leiden, The Netherlands.

(b) *Preparation of genomic DNA*

Two cubic millimetres of foot tissue of a single snail were ground in 200 µl of sucrose buffer (0.1 M Tris, 0.02 M NaCl, 0.2 M sucrose and 0.05 M EDTA) and centrifuged. The pellet was incubated at 65 °C for 60 min in 200 µl SDS buffer (0.02 M Tris, 0.01 M EDTA and 1.25% SDS), 15 µl of cold 5 M potassium acetate was added and the mixture was incubated on ice for 60 min and centrifuged. The DNA was precipitated from the

supernatant by the addition of two volumes of 100% ethanol and incubation at –20 °C for 30 min. The DNA was dried, treated with 200 ng of RNase and dissolved in 30 µl of ddH₂O. Extractions were carried out for 18–33 individuals per sample, with the exception of the *A. h. harmonia* samples, from which a single individual was studied as a reference.

(c) *Polymerase chain reaction, restriction endonuclease fingerprinting and sequencing of the CaM intron*

First, the intron (situated between exons III and IV; Swanson *et al.* 1990) was amplified in *A. h. harmonia* using an exon-primed, intron-crossing polymerase chain reaction (PCR), with the primer Cal-1 located at the 3'-end of exon III (5'-GCCGAGCTGCARGAYATGATCAA-3') and the primer Cal-2 located at the 5'-end of exon IV (5'-GTGTCCTTCATTTTNCCKTGCCATCAT-3') from the nDNA insect primer kit (B. Crespi and J. Hobbs, University of British Columbia, Vancouver, Canada). The amplification product was cloned in a pGEM T-vector (Promega, Madison, WI, USA) and sequenced in both directions on an automated ABI sequencer using the T7 and SP6 priming sites and standard protocols. The following internal primers were designed on the basis of this sequence: Cam-1 (5'-ACATGATCAATGAAGTGGATGC-3') and Cam-2 (5'-MWYMWKGTGAGGAATTCTGG-3'). Fifteen picomoles of each of these primers was then used in amplification reactions of 50 µl, with a final Mg²⁺ concentration of 2.8 mM, 5 µl of undiluted genomic DNA and otherwise standard conditions. The PCR programme consisted of a denaturation step of 2 min at 94 °C, followed by 30 cycles of 40 s at 92 °C, 40 s at 58 °C and 1 min at 72 °C and ended with a final extension step of 5 min at 72 °C. Restriction endonuclease fingerprinting (REF) (Liu & Sommer 1995) was performed as follows: 10 µl of PCR product was digested with *AccI* (Boehringer-Mannheim), resulting in three fragments of ca. 150, 345 and 385 bp in length. (This restriction enzyme was chosen on the basis of the *A. h. harmonia* sequence.) The fragments were denatured at 95 °C for 5 min, then quenched on ice water and run for 10 h on a 7.5% polyacrylamide gel with 7% glycerol (180 V at 7 °C). Samples of parental and hybrid origin were always run together on the same gel. Finally, the gel was silver stained (Sambrook *et al.* 1989). For the detection of haplotypes, only the two smallest fragments gave sufficient resolution, so we ignored the largest fragment. Detected haplotypes were directly sequenced in one to ten homozygous individuals, using Cam-1 as the sequencing primer. Two haplotypes that were present only in heterozygotes were cloned instead. The correct clones were identified by performing the Cam-1–Cam-2 PCR reaction on transformed *Escherichia coli* colonies, after which the PCR products were screened by REF as described above and sequenced in both directions using the T7 and SP6 priming sites on the vector.

(d) *Analysis*

The haplotype frequencies for *CaM* were calculated in BIOSYS-1 (Swofford 1989). Conformance to the Hardy–Weinberg equilibrium was tested using the exact probability test provided by BIOSYS-1. The sequential Bonferroni correction (Holm 1979) was applied to all six tests. The sequences (GenBank accession numbers AF132310–AF132317) were aligned manually. The genealogies of the haplotypes were determined using a parsimony approach in PAUP*4.0b1 (Swofford 1999). Analysis was performed with the branch-and-bound

Table 1. Genotype frequencies at each of the six localities where samples were taken

	AW	AE	CW	CE	HW	HE
AA	10	27	4	9	—	—
AB	4	—	4	—	—	—
AC	—	1	5	3	—	—
AD	2	—	2	5	—	—
AE	—	—	—	1	—	—
AF	1	—	—	1	—	—
AG	—	1	—	—	—	—
BC	—	—	1	—	—	—
CC	—	—	9	2	—	—
CD	1	—	1	2	—	—
CE	—	—	—	4	—	—
DD	—	—	2	—	12	18
DE	—	—	2	1	8	3
DF	—	—	—	—	1	2
EE	—	—	—	1	6	1
EF	—	—	—	2	5	1
FF	—	—	—	2	1	2

algorithm, treating a gap as a fifth character state. The trees were rooted with *A. h. harmonia*.

3. RESULTS AND DISCUSSION

REF revealed seven haplotypes (table 1), termed *A–G*, which belong to two distinct monophyletic groups: *A + B* and *C + D + E + F + G* (figure 2). Deviation from Hardy–Weinberg equilibrium was found in sample *CW* ($p=0.007$). In the pure *A. h. aphrodite* samples, the *A* haplotype dominated (0.75 in locality *AW* and 0.97 in locality *AE*), with the *B*, *C*, *D*, *F* and *G* haplotypes at low to very low frequencies. In the pure *A. h. holtzi* samples, *D* was the commonest haplotype (0.50 in locality *HW* and 0.76 in locality *HE*) and *E* and *F* were relatively minor variants. In the hybrid populations, a mixture of all parental haplotypes was found, as expected. Interestingly, though, the *C* haplotype, which was found at very low frequencies in *A. h. aphrodite* (0.03 in locality *AW* and 0.02 in locality *AE*) and not at all in *A. h. holtzi*, reached frequencies of 0.20 and 0.42 in localities *CW* and *CE*, respectively (figure 3). The sequences (table 2) and their genealogy (figure 2) reveal that the *C* haplotype is related to the other haplotypes by simple nucleotide substitutions and that it carries a number of unique mutations.

The situation for this intron is surprisingly similar to that seen in allozymes. The single variant *C*, which is rare elsewhere, reaches relatively high frequencies in the centre of the hybrid zone. There is no indication of increased mutation rates, since no additional haplotypes are found in the hybrid *CE* and *CW* populations. Neither can *C* be considered a recombinant, because it possesses nucleotides at positions 349 and 467, which are not shared by any of the other haplotypes. These apomorphies are found consistently in all individuals sequenced for the *C* haplotype.

Drift is an unlikely cause of the observed increase in the frequency of *C*. Not only is the same haplotype increased in two populations separated by more than 2 km (whereas migration in *Albinaria* is in the order of

Figure 2. Genealogical relationships between the seven haplotypes of the *CaM* intron found in the hybrid zone. The two most parsimonious trees are shown (length = 24 steps and consistency index (CI) = 0.92; Kluge & Farris 1969).

Figure 3. Haplotype frequencies at each locality. Note the increase in haplotype *C* at locations *CW* and *CE*. Other abbreviations as in the main text.

3 m per generation²; Schilthuizen & Lombaerts 1994), but demes in *Albinaria* normally experience sufficient gene flow to prevent genetic differentiation due to drift (Schilthuizen & Lombaerts 1994). Selection remains as the only plausible explanation. Since this is a non-coding region, selection must act on a locus closely linked to it, either the *CaM* exons themselves or a neighbouring gene.

Table 2. *Variable positions at the seven haplotypes detected in the hybrid zone and the reference sequence (Albinaria hippolyti harmonia) in n individuals sequenced*

(The region runs from the 3'-end of the preceding exon (position 0) to the second *AccI* site (position 474). Due to sequencing difficulties, the 5'-end of the *B* haplotype could not be determined accurately.)

	24	36	73	75	87	174	217	224	241	259	260	268	288	304	349	396	398	402	434	459	467	<i>n</i>
<i>harm</i>	C	A	T	T	T	G	C	C	A	—	—	G	G	G	A	A	A	A	T	C	T	1
<i>A</i>	T	A	G	A	G	G	T	A	A	A	G	G	G	T	A	A	C	G	T	T	T	10
<i>B</i>	?	?	?	?	?	?	T	A	C	—	G	G	—	T	A	A	C	G	C	T	T	1
<i>C</i>	C	A	G	A	G	G	C	A	A	A	A	A	G	T	C	A	C	A	T	T	G	5
<i>D</i>	C	A	G	A	G	A	C	A	A	A	—	A	G	T	A	A	C	A	T	T	T	3
<i>E</i>	C	A	G	A	G	A	C	A	A	—	—	A	G	T	A	A	C	A	T	T	T	2
<i>F</i>	C	G	G	A	G	A	C	A	A	A	—	A	G	T	A	A	C	A	T	T	T	3
<i>G</i>	C	A	G	A	G	A	C	A	A	A	—	A	G	T	A	G	C	A	T	T	T	1

Some indication of selection may be found in the CW population, which shows a strong deviation from Hardy–Weinberg equilibrium, mainly caused by an overrepresentation of *CC* homozygotes. However, such a deviation is not seen in the CE population.

There could be many opportunities for markers to hitchhike with selected loci in hybrid zones. Studies of natural and experimental hybridization have shown that the genes responsible for reproductive isolation are both numerous and scattered widely over the genome (figures of around 100 loci have been calculated for species pairs in *Drosophila*, toads and grasshoppers; Barton & Hewitt 1981; Szymura & Barton 1986, 1991; Palopoli & Wu 1994). It is likely that there will be genetic variation at these loci for the degree of reproductive isolation. In fact, Rieseberg *et al.* (1996) discovered that hybrid speciation in sunflowers consistently results in particular multilocus genotypes, which suggests that certain combinations of parental alleles increase hybrid fitness. Presumably, therefore, in hybrid zones many genes may come under strong selection, with hitchhiking of any associated markers.

The rare allele phenomenon might be the manifestation of such selection for genes which enhance hybrid fitness. However, there is no reason why the hitchhiking markers need to be rare alleles. The same might happen with markers that are already common in one of the parental taxa, but these may not readily be noticed because they merely result in a shift of the marker cline towards the opposite side of the hybrid zone. As a matter of fact, a few examples of such cline shifts have been observed in hybrid zones, for both allozymes (Hunt & Selander 1973; Schilthuizen 1995) and chromosomal markers (Hewitt 1993; Searle 1993).

The associations between selected loci and markers will eventually be broken up by recombination in the parental populations. As a result, the patterns of hybridzymes which are set up after the initial contact between the two hybridizing taxa are probably transient and subject to cycles of increase in certain alleles, decrease in the same alleles and replacement by newly arisen alleles. This may explain why some hybridzymes cannot be demonstrated outside of the hybrid zone; these might be the result of mutations that took place in a hybrid individual and were subsequently locally selected. It might be interesting to monitor hybridzyme prevalences over many

generations to see whether and how rapidly such turn-over takes place.

In conclusion, we believe that the rare allele phenomenon (and similar patterns in karyotypic markers; see Serrano *et al.* 1996) might be more relevant than has been previously appreciated. These markers could serve as an addition to traditional hybrid zone analysis in revealing the nature and locality of the genes responsible for reproductive isolation. This may be particularly useful in groups that defy standard genetic analysis because they are difficult to culture in the laboratory.

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