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Studies of mitochondrial DNA, allozyme and morphometric variation in a house mouse hybrid zone

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Summary

An unusual chromosomal hybrid zone of the house mouse, *Mus musculus domesticus*, exists in Upper Valtellina, Northern Italy, consisting of four Robertsonian (Rb) races and the standard (all-acrocentric, or $2n = 40$) race, all hybridizing freely within 10 km². The hybrid zone in Valtellina provides an excellent opportunity to study the role of Rb fusions in reproductive isolation and speciation. This hybrid zone has already been well studied for the distribution of Rb fusions and the fertility of hybrids, but in order to understand the dynamics of the zone, a basic understanding of the origin and genetic similarity of the chromosomal races is necessary. This paper presents the results of three different methods of measuring genetic differentiation: multivariate analysis of morphological traits and analyses of allozyme variation and mitochondrial DNA sequences. The standard race is clearly distinguishable from the three Rb races by all three methods, but the Rb races are not distinguishable from one another. This provides strong evidence for our previous suggestions that the well-established Rb races in Valtellina are closely related, and that the standard race was introduced into the valley more recently from a distant source. The fact that the Rb races are indistinguishable is also consistent with our hypothesis that a within-village speciation event involving two of the races (Hauffe & Searle, 1992) was a recent occurrence. The low level of allozyme heterozygosity among the Rb races suggests that these populations are the products of at least one bottleneck. The present article substantially extends earlier studies and provides the first detailed morphological and molecular analysis of this complex hybrid zone.

1. Introduction

Robertsonian (Rb) races are populations homozygous for a set of metacentric chromosomes: the result of repeated Rb (centric) fusions. In the house mouse, *Mus musculus domesticus*, the standard karyotype is 20 pairs of acrocentric chromosomes, but diploid numbers vary from 22 to 38 across its range in Europe and North Africa (Sage *et al.*, 1993; Nachman & Searle, 1995). Groups of contiguous populations that share the same homozygous set of Rb fusions are referred to as 'races' (Hausser *et al.*, 1994). The variation resulting from Rb fusions is probably also augmented by whole-arm reciprocal translocations and generation of hybrid races (Hauffe & Piálek, 1997; Piálek *et al.*, 2001). Although there may be some sharing of metacentrics between different Rb races in the house mouse (due to common ancestry or convergent evolution), the set of metacentrics is almost always unique for any particular Rb race. Hybrids between Rb races and the standard race or between two Rb races have been shown to suffer reduced fertility and sometimes complete sterility in the house mouse, resulting from non-disjunction or other abnormalities of heterozygous configurations at meiosis I (Searle, 1993). The occurrence of such clear-cut hybrid unfitness has made the chromosomal races in the house mouse an important model for the study of speciation (King, 1993). Over the last 12 years these speciation studies have largely involved the analysis of hybridization between karyotypic races of house mice in nature.

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A particular Rb race generally has a very limited geographic distribution. For example, as many as six different Rb races have recently been described on the small Atlantic island of Madeira (Britton-Davidian *et al*., 2000). In mainland areas, the Rb races occur singly or in groups surrounded by the much more widely distributed standard race. The large majority of studies of chromosomal hybrid zones in the house mouse have focused on contacts between a Rb race and the standard race, such as those in Belgium (Hübner & Koulischer, 1990; Bauchau *et al*., 1990), Denmark (Nance *et al*., 1990; Fel-Clair *et al*., 1996, 1998), northern Scotland (Searle, 1991; Wallace *et al*., 1992; Searle *et al*., 1993), central Italy (Castiglia & Capanna, 1999, 2000), northern Spain (Gündüz *et al*., 2001) and Tunisia (Saïd & Britton-Davidian 1991; Saïd *et al*., 1993, 1999). These studies have shown that hybrid zones between the standard race and a Rb race with a high diploid number (i.e. characterized by few metacentric chromosomes) tend to be weak genetic barriers. For example, the metacentric clines at the contact between the John o'Groats ($2n = 32$) and standard races in Scotland are separated from each other in the hybrid zone, such that highly heterozygous low-fertility hybrids are not produced (Searle *et al*., 1993). Those hybrid zones involving the standard race and a Rb race with low diploid number/many metacentric chromosomes can be much stronger genetic barriers. For example, the Tunisian hybrid zone between the Monastir ($2n = 22$) and standard race is very narrow, prompting discussion on whether this is a site of 'chromosomal speciation' (Saïd *et al*., 1999), i.e. speciation promoted by substantial chromosomal divergence (King, 1993). This discussion could be particularly informed because of the combination of data available for the Tunisian zone: on karyotypic variation, hybrid fertility, morphological and molecular variation (allozymes, mtDNA) (review: Saïd *et al*., 1999).

Most relevant to the possibility of chromosomal speciation in the house mouse is the analysis of hybrid zones between different Rb races, as hybrids produced in such contacts are expected to be particularly unfit on chromosomal grounds (Searle, 1993). Since 1989, we have been studying a hybrid zone in northern Italy, which involves both contacts between different Rb races and between Rb races and the standard race (Hauffe & Searle, 1992, 1993, 1998; Fraguedakis-Tsolis *et al*., 1997; Hauffe *et al*., 2000; Piálek *et al*., 2001). These studies have mostly involved karyotypic surveys and fertility studies and have been hampered by a lack of data on morphology and molecular markers, for the interpretation of gene flow and genetic distinctiveness of the races. Here we present data on mandible and external morphology, allozymes and mitochondrial D-loop sequences for this hybrid zone, to rectify this situation.

The hybrid zone is located in Upper Valtellina, which includes the north-eastern reaches of Valtellina, a long narrow alpine valley extending from Lake Como to Bormio. The valley is flanked by mountains 2000–3000 m high. Mouse populations are restricted to habitations up to 900 m altitude; thus, natural dispersal is limited to a north-east/south-west direction along the valley floor where most villages are situated. Several villages are also isolated from others by forest, gorges and/or the fast-flowing alpine River Adda (Fig. 1). Mice could conceivably also be transported by humans mainly from the west (Lower Valtellina to Lake Como) and north (Val Poschiavo, Switzerland; see Fig. 1), with minor mountain passes to and from north-eastern Italy and southern Switzerland. The mice in Valtellina are found exclusively in old buildings, especially those housing domestic animals usually found in the historical centres of present villages. The five karyotypic races within the Upper Valtellina hybrid zone are within an area of 10 km^2 (Fig. 1; Hauffe & Searle, 1993).

With its multiple contacts in a small geographic area, the Upper Valtellina hybrid zone provides an excellent opportunity to study the role of Rb fusions in speciation. In fact, in the early 1980s, Capanna and Corti reported that in one small village, Migiondo (see Fig. 1), two Robertsonian races, 24UV and 26POS, were found to be sympatric, but not successfully interbreeding (Capanna & Corti, 1982). The fact that natural hybrids exist in Sommacologna (less than 1 km from Migiondo) indicates that there are no such barriers to interbreeding elsewhere; therefore, it most probably evolved in Migiondo itself. This situation presented the interesting possibility of a reinforcement event, probably favoured by the relative isolation of the village (Hauffe & Searle, 1992, 1993). Despite the fact that in 1992 the 24UV race was shown to be extinct in Migiondo (Hauffe & Searle, 1992), we are still investigating in detail the evolution of assortative mating using individuals from Migiondo and the surrounding villages (see Piálek *et al*., 2001).

The morphological and molecular studies of Upper Valtellina mice that we will describe here add to a small existing base of information. Thorpe *et al*. (1982) compared three of the Valtellina races by multivariate morphometrics (the other two races were undiscovered at that time), but only in one population each; there are mtDNA sequences for three individuals of two of the races found in Valtellina within Europe-wide surveys (Prager *et al*., 1993; Nachman *et al*., 1994), and there have been limited studies in Valtellina involving allozyme loci (Capanna *et al*., 1985; Fraguedakis-Tsolis *et al*., 1997). The present article substantially extends these earlier studies of this complex hybrid zone and provides the first detailed analysis using genetical characters different from the chromosomal markers used to define the zone.

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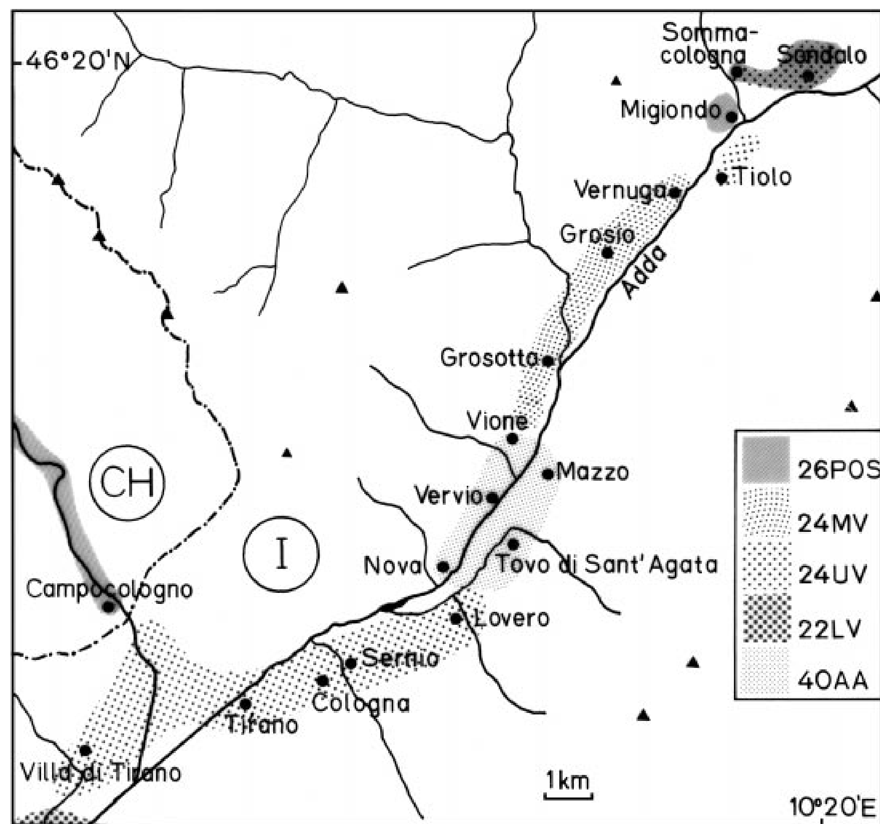

Fig. 1. Map of Upper Valtellina (Lombardy, Italy). Centres of villages are marked by filled circles, mountain peaks by filled triangles. Chromosomal races which dominate each village are indicated by shading (see legend): 26POS, Poschiavo race ($2n = 26$: carrying Rb fusions Rb(1.3), Rb(4.6), Rb(5.15), Rb(8.12), Rb(9.14), Rb(11.13), Rb(16.17)); 24MV, Mid Valtellina race ($2n = 24$: Rb(1.3), Rb(4.6), Rb(5.15), Rb(7.18), Rb(8.12), Rb(9.14), Rb(11.13), Rb(16.17)); 24UV, Upper Valtellina race ($2n = 24$: Rb(1.3), Rb(2.8), Rb(4.6), Rb(5.15), Rb(9.14), Rb(10.12), Rb(11.13), Rb(16.17)); 22LV, Lower Valtellina race ($2n = 22$: Rb(1.3), Rb(2.8), Rb(4.6), Rb(5.15), Rb(7.18), Rb(9.14), Rb(10.12), Rb(11.13), Rb(16.17)); 40AA, all-acrocentric race (no fusions). Reprinted, with permission, from Hauffe & Searle (1998 © the Genetics Society of America).

2. Materials and methods

(i) *Sample collection*

Wild mice were live-trapped in the autumn of 1989, 1990 and 1991 from farms in 17 villages in Upper Valtellina, Italy (Fig. 1). All specimens were taken live to the Laboratorio Centrale of the Eugenio Morelli Hospital, Sondalo, where external morphological measurements were recorded (see below) and karyotypes were determined (Hauffe & Searle, 1993; H. C. Hauffe, unpublished data). Individuals were assigned to one of seven groups according to karyotype. Five groups have karyotypes of chromosomal races: 40AA, 22LV, 24UV, 24MV and 26POS (see footnote in Fig. 1); two groups have hybrid karyotypes: AA × Rb and Rb × Rb. 'AA × Rb' are single or multiple simple heterozygotes most likely produced by a cross between 40AA and a Rb race, or a higher-generation hybridization event ($2n = 28$–39). 'Rb × Rb' hybrids are heterozygotes resulting from the interbreeding of mice from two Rb races ($2n = 23$–25). Bodies, tail tips and tissue samples (kidney, liver, heart, spleen and muscle) of all house mice captured were kept at −20 °C and then transported on ice to the UK and stored at −20 °C (whole animal) or −80 °C (tissues).

(ii) *Morphometrics*

Mandible morphology was studied for 98 house mice collected in the autumn of 1990. The mandibles were taken from frozen specimens, the heads of which were thawed for 10 min and the skin and underlying fat removed. The heads were boiled vigorously for 45 s in tap water, cooled, and then incubated at 37 °C in 20 ml of a saturated solution of NaCl and 'Biotex' (biological laundry powder) for 3 days. After incubation, the bones were separated easily by placing the skull in a sieve and applying a gentle spray of tap water. The mandibles were left to dry for 2 days at room temperature. Temporal variation in mandible morphology was minimized by studying animals collected in one season (after Thorpe, 1976). Although analysis of morphological variation should be restricted to mice of an identical growth stage (Thorpe & Leamy, 1983), in practice, ontogenetic variation

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can be minimized by avoiding the youngest animals with the fastest growth (Green & Fekete, 1933; Lovell *et al*., 1984). Thus, in this study, each specimen was assigned to dental wear stages 1 to 7 by the method of Lidicker (1966) and only individuals older than 2 months of age (stage 3) were used (after Davis, 1983). Eighty-nine samples were assigned to stage 3 or above. The mandible was measured according to Festing (1972) except that the mandible was placed with the condyle against the vertical slide (to avoid variation due to tooth wear). Festing's characters #4 and #8 were not used, as tooth wear or tooth rocking can affect these measurements (Davis, 1983); therefore, a total of 11 measurements were made per mandible. To avoid variation introduced by asymmetry, only the right mandible was used (Leamy, 1984). All samples were measured on the same day; 20 samples were re-measured the following day and error was established to be less than 1%.

External morphological measurements included body weight, pelt colour, and body, tail, left ear and left foot length. The pelt colour was judged on a scale of 1 to 10, based on the lightest and darkest pelt colours observed in *M. m. domesticus* in Upper Valtellina; preserved pelts were used to standardize comparison. Individuals with partially missing tails or ragged ears were not included in this analysis. Measurements were complete for 82 mice (2 or more months old, as judged by tooth wear, size and/or weight). In preliminary analysis, sexual dimorphism within karyotypic groups was not found for any measurement (MANOVA $P > 0{\cdot}05$); therefore, measurements of males and females from the same karyotypic group were pooled.

In order to assess relationships between groups, canonical variate analysis (CVA) was performed independently for mandible and external measurements using Numerical Taxonomy System (NTSYSpc, version 2.00, Applied Biostatistics, 1997; Rohlf, 1990). It was not possible to extract a 'size' vector from the data since the sign and magnitude of the first eigenvector for each group were not similar (Macholán, 1996). To examine the distance between the centroids of different groups, Hotelling's T^2 was calculated from the generalized distances (D) generated by CVA, after Marcus (1993). The level of significance of the resulting F values was determined for pooled samples with a Bonferroni-type adjustment as suggested by these authors and minimum F values were generated using $\alpha = 0{\cdot}003$ and df = 11, 73 for mandible measurements and df = 6, 71 for external morphological measurements.

(iii) *Mitochondrial DNA sequencing*

Genomic DNA was extracted from 2 mm tail samples of 33 house mice captured in 1990 following the method in Bilton *et al*. (1998). Only 40AA and homozygous Rb mice were used. A 724 bp portion of the mtDNA was amplified by the polymerase chain reaction (PCR) using a Perkin-Elmer Cetus thermal cycler with hot bonnet, and the 'universal' primers L15774 (Shields & Kocher, 1991) and H16498 (P. Taberlet, personal communication). Thirty amplification cycles with 93 °C for 50s, 50° for 1 min, 72 °C for 2 min were used. Double-stranded PCR products were purified with QIAquick spin columns (Qiagen). Approximately 125 ng of PCR product was prepared with the primers using a Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems, ABI) before being directly sequenced on an ABI 373A automated sequencer. The PCR product was sequenced from both ends and all DNA samples were sequenced twice to exclude reading errors and contamination of PCR products. A 210 bp fragment was generated from the L15774 end of the PCR product and included part of the cytochrome *b*, tRNA Thr and tRNAPro genes (nucleotide positions 15192–15401; Bibb *et al*., 1981). A 142 bp fragment was generated from the H16498 end of the PCR fragment that was entirely within the D-loop (nucleotide positions 15586–15727; Bibb *et al*., 1981). For each individual, the 210 bp and 142 bp fragments were combined for a total of 352 base pairs for phylogenetic analysis. Sequences were aligned using the Genetics Computer Group package (Devereux *et al*., 1984).

(iv) *Allozyme study*

For the allozyme study, complete data were obtained for 129 house mice pooled from 1989, 1990 and 1991. The allozymes were separated by electrophoresis on Titan III cellulose acetate plates (Helena Laboratory, Beaumont, Texas, USA), with runs of 15–45 min at 200 V, following the methodology of Searle (1985) and the histological staining procedure of Harris & Hopkinson (1976). The enzymes that could readily be scored from house mouse tissue (specified) with this system were: acid phosphatase (ACP, EC 3.1.3.2, kidney), aconitase (ACO, EC 4.2.1.3, heart), adenosine deaminase (ADA, EC 3.5.4.4, spleen), adenylate kinase (AK, EC 2.7.4.3, heart), amylase (AMY, EC 3.2.1.1, kidney), creatine kinase (CK, EC 2.7.3.2, heart), glucose-6-phosphate dehydrogenase (GPD, EC 1.1.1.49, kidney), glucose phosphate isomerase (GPI, EC 5.3.1.9, kidney), glutamate oxaloacetate transaminase (GOT, EC 2.6.1.1, kidney), isocitrate dehydrogenase (IDH, EC 1.1.1.41, kidney), lactate dehydrogenase (LDH, EC 1.1.1.27, kidney), malate dehydrogenase (MDH, EC 1.1.1.37, kidney), malic enzyme (ME, EC 1.1.1.40, liver), mannose phosphate isomerase (MPI, EC 5.3.1.8, kidney), nucleoside phosphorylase (NP, EC 2.4.2.1, kidney) and phosphoglucomutase (PGM, EC 2.7.5.1, kidney). The electro-

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phoretic running buffers were 40 mM Tris–10 mM citrate (pH 7·6) for ACO, AK, CK, IDH, MDH and ME, 25 mM Tris–190 mM glycine (pH 8·5) for GPD, GPI, LDH, MPI, NP and PGM, and 40 mM phosphate (pH 6·3) for ACP, ADA, AMY and GOT. Allozyme loci and alleles were named according the standard nomenclature developed for the laboratory mouse (Lyon *et al*., 1996); use was made of tissue samples from mouse inbred strains to confirm identification. Any loci or alleles of uncertain homology with previously described markers were given a provisional nomenclature.

Unbiased average heterozygosities were estimated from the complete allozyme data by the method of Nei (1987). Population structure was analysed by the approach of Excoffier *et al*. (1992) and Michalakis & Excoffier (1996). The Arlequin v.1.1 package (Schneider *et al*., 1997) was used to calculate pairwise Φ_{ST} values among populations based on the allozyme allele frequencies (analogous to F_{ST}), and to carry out a permutation test for their significance. A nested analysis of molecular variation (AMOVA) was also conducted using Arlequin.

3. Results

(i) *Morphometrics*

The results of CVA (Table 1) clearly show for both mandible measurements and external morphological measurements that the three Rb races (26POS, 24UV

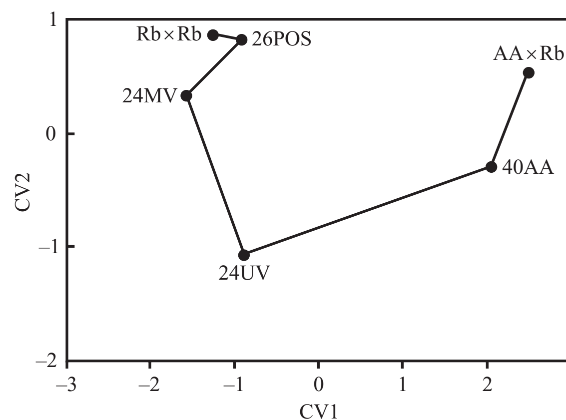

Fig. 2. Plot of the first two canonical variate scores for CVA on mandible measurements. Groups are joined by the minimum-spanning tree.

and 24MV) and their hybrids (Rb × Rb) are morphologically distinct from the all-acrocentric race (40AA) and AA × Rb hybrids: all *F* values for comparisons between Rb races and their hybrids are not significant, while all comparisons between these groups and 40AA and AA × Rb are highly significant. 40AA and AA × Rb individuals are morphologically indistinguishable. It is not surprising that the AA × Rb hybrids are morphologically more similar to 40AA than to the Rb races: these hybrids are not first-generation hybrids but the result of hybrids backcrossed to 40AA (because the AA × Rb individuals were $2n = 35–39$ for the morphometric analysis). The three Rb races and

Table 1. *Generalized distances,* T^2 *values and* F *values for canonical variate analysis of mandible measurements*[a] (*below diagonal*) *and external measurements*[b] (*above diagonal*)

Race[c] (N)		40AA (16)	26POS (10)	24UV (23)	24MV (11)	AA × Rb (11)	Rb × Rb (11)
40AA (17)	*D*	–	3·93	3·16	3·74	1·43	3·07
	T^2	–	95·02	93·89	91·32	13·35	61·36
	F	–	**15·20**	**15·02**	**14·61**	**(2·32)**	**9·82**
26POS (13)	*D*	3·36	–	1·11	1·24	3·58	2·09
	T^2	83·24	–	8·59	8·00	66·94	22·97
	F	**6·66**	–	**(1·37)**	**(1·28)**	**10·71**	**(3·68)**
24UV (12)	*D*	3·26	2·05	–	0·98	2·75	1·17
	T^2	105·58	35·46	–	7·08	56·36	10·16
	F	**8·45**	**(2·84)**	–	**(1·13)**	**9·02**	**(1·63)**
24MV (12)	*D*	3·87	1·47	1·88	–	3·32	1·30
	T^2	105·52	13·52	28·23	–	60·55	9·35
	F	**8·44**	**(1·08)**	**(2·26)**	–	**9·69**	**(1·50)**
AA × Rb (12)	*D*	1·67	3·64	3·80	4·26	–	2·43
	T^2	19·53	82·88	115·56	109·14	–	32·55
	F	**(1·56)**	**6·63**	**9·24**	**8·73**	–	**5·21**
Rb × Rb (11)	*D*	3·77	1·40	2·10	1·58	3·90	–
	T^2	94·77	11·64	33·35	14·27	87·38	–
	F	**7·58**	**(0·93)**	**(2·67)**	**(1·14)**	**6·99**	–

[a] Values of $F_{11,73} > 2·92$ are significant at $P = 0·05$ (with Bonferroni-type adjustment; Marcus, 1993). Non-significant values shown in parentheses.
[b] Values of $F_{6,71} > 3·70$ are significant at $P = 0·05$ (with Bonferroni-type adjustment; Marcus, 1993). Non-significant values shown in parentheses.
[c] See Fig. 1.

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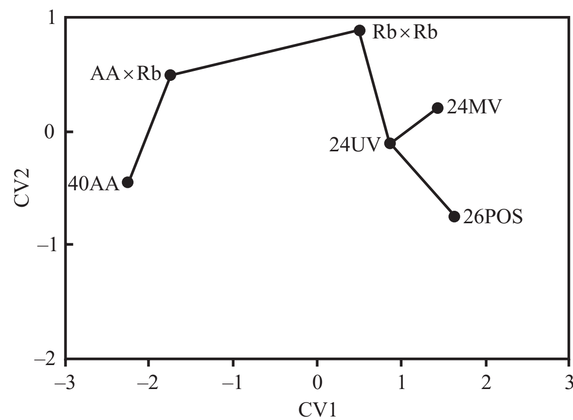

Fig. 3. Plot of the first two canonical variate scores for CVA on external morphological measurements. Groups are joined by the minimum-spanning tree.

Rb×Rb individuals are also morphologically indistinguishable.

These results can be visualized in Fig. 2 (mandible measurements) and Fig. 3 (external morphological measurements), which show minimum spanning trees joining group centroids in plots of the first two canonical variates. Both trees demonstrate that the 40AA and AA×Rb animals are distinct from the other races and Rb×Rb hybrids. The standard measurements of Rb×Rb mice are more similar to those of AA×Rb mice than are their mandibles.

(ii) *Mitochondrial DNA sequencing*

Polymorphic nucleotide sites found in the 352 base pair sequence in all 33 animals are shown in Table 2. Eight polymorphic sites (2·3%) were found. One silent base change was detected in the cytochrome *b* gene of the mtDNA; three base changes were noted in the tRNAThr gene and four in the control region. As expected for mammals (e.g. Taberlet *et al*., 1994) most base changes are transitions (5 of 8, 63%). The range of pairwise distances between haplotypes is 0–1·1%.

The pattern of polymorphism that emerges is quite simple: nucleotide site 15597 (within the D-loop) divides the sample into two main groups, one containing all the 40AA individuals characterized by a T, and one containing individuals of the four Rb races 22LV, 24UV, 24MV and 26POS, characterized by a C. There are three exceptions: samples Migiondo 19 (26POS), Prada 2 and Prada 3 (both 24MV), are all characterized by a T. Only one other Rb race individual out of 25 shows additional polymorphism: the single 22LV individual (Grosotto 16). Four out of

Table 2. *Polymorphic nucleotide sites in the mtDNA of the 33 individuals analysed*

Individual[a]	Race[b]	Polymorphic sites[c]							
		15257 A	363 G	381 C	383 C	586 T	597 C	704 A	718 A
Sondalo 7	26POS	.	.	.	.	.	.	.	.
Sommacologna 4	24UV	.	.	.	.	.	.	.	.
Migiondo 2, 4, 7, 8, 9, 13, 17, 21, 25	26POS	.	.	.	.	.	.	.	.
Migiondo 19	26POS	.	.	.	.	.	T	.	.
Sontiolo 4	24UV	.	.	.	.	.	.	.	.
Tiolo 4, 5	24UV	.	.	.	.	.	.	.	.
Grosotto 14	24MV	.	.	.	.	.	.	.	.
Grosotto 16	22LV	.	.	.	.	C	.	.	.
Prada 2, 3	24MV	.	A	.	.	.	T	.	T
Mazzo 4, 16	**40AA**	.	.	.	.	.	**T**	.	.
Mazzo 7	**40AA**	.	.	.	**A**	.	**T**	.	.
Tovo 1	**40AA**	.	.	.	.	.	**T**	**G**	.
Tovo 4, 8, 14	**40AA**	.	.	.	.	.	**T**	.	.
Tovo 5	**40AA**	.	.	**A**	.	.	**T**	.	.
Tovo 12	**40AA**	**G**	.	.	.	.	**T**	.	.
Biolo 1, 2	24UV	.	.	.	.	.	.	.	.
Sernio 1, 4	24UV	.	.	.	.	.	.	.	.
Villa di Tirano 5	24UV	.	.	.	.	.	.	.	.

[a] Name of village and number of individual animal; villages are listed geographically (see Fig. 1).
[b] See Table 1 for abbreviations; 40AA individuals are indicated in bold.
[c] Numbers refer to nucleotide positions from the published sequence of Bibb *et al*. (1981). The consensus sequence of all individuals is given; dots indicate agreement with the consensus.

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Table 3. *Frequency data for polymorphic allozyme loci for all populations sampled in Upper Valtellina (listed in geographic sequence: see Fig. 1). Those populations identified as being dominated by the 40AA race and AA × Rb hybrids with high chromosome numbers are highlighted in bold (these are referred to as the 'AA – dominated' localities in the text, as opposed to the other, 'Rb-dominated' localities)*

			Allele[a] frequencies											
			Amy1		*Got2*		*Idh1*		*LdrX*		*Mod1*			
Site name	Chromosomal characteristics[a]	*n*	*u*	*v*	*a*	*b*	*a*	*b*	*a*[b]	*b*	*a*	*c*	*n2*[b]	*H*[c]
Sondalo	24UV, 26POS, Rb × Rb	11	0·136	0·864	1	0	0	1	1	0	1	0	0	0·012
Sommacologna	24UV, 26POS, Rb × Rb	11	0	1	1	0	0	1	1	0	0·955	0·045	0	0·004
Migiondo	26POS	22	0	1	1	0	0·341	0·659	1	0	0	0·574	0·426	0·046
Sontiolo	24UV	4	0	1	1	0	0	1	1	0	1	0	0	0
Tiolo	24UV	7	0	1	1	0	0	1	1	0	1	0	0	0
Lago	24UV	2	0	1	1	0	0	1	1	0	1	0	0	0
Grosio	24MV, Rb × Rb	9	0	1	1	0	0·056	0.944	1	0	1	0	0	0.005
Grosotto	22LV, 24MV, Rb × Rb	16	0	1	1	0	0·031	0·969	1	0	0·875	0·125	0	0·014
Prada	24MV	5	0	1	1	0	0	1	1	0	1	0	0	0
Vione	26POS, AA × Rb	3	0	1	0·667	0·333	0·500	0·500	1	0	1	0	0	0·054
Mazzo	**40AA, AA × Rb**	**12**	**0·958**	**0·042**	**0·500**	**0·500**	**0·625**	**0·375**	**0·592**	**0·408**	**1**	**0**	**0**	**0·076**
Vervio	**40AA**	**1**	**1**	**0**	**0·500**	**0·500**	**0**	**1**	**1**	**0**	**1**	**0**	**0**	**0·048**
Tovo	**40AA, AA × Rb**	**13**	**0·962**	**0·038**	**0·423**	**0·577**	**0·115**	**0·885**	**0·380**	**0·620**	**1**	**0**	**0**	**0·059**
Nova	**AA × Rb**	**2**	**0·500**	**0·500**	**1**	**0**	**0·750**	**0·250**	**1**	**0**	**1**	**0**	**0**	**0·056**
Lovero (farm 34)	**AA × Rb**	**1**	**1**	**0**	**0**	**1**	**0**	**1**	**1**	**0**	**1**	**0**	**0**	**0**
Lovero (rest)	24UV	3	0	1	1	0	0	1	1	0	1	0	0	0
Sernio	24UV	5	1	0	1	0	0	1	1	0	1	0	0	0
Biolo	24UV	2	0	1	1	0	0	1	1	0	1	0	0	0

[a] See text for nomenclature relating to chromosomal characteristics of the populations and to allozyme loci and alleles.
[b] For recessive alleles (*LdrX*[a], *Mod1*[n2]), frequencies were estimated assuming that the recessives were only present in the populations where they were detected and that within those populations, the recessives were in Hardy–Weinberg equilibrium with the dominant.
[c] Unbiased estimates of average heterozygosity (*H*) are based on the combined data of the polymorphic loci and the 16 monomorphic loci screened (see text).

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Table 4. *Estimates of pairwise Φ_{ST} among populations, based on allozyme allele frequencies*

	So	Som	Mig	Son	Tio	Lag	Gr	Gro	Pra	Vio	Maz	Ver	Tov	Nov	Lo	Lov	Ser
Sommacologna	0·095																
Migiondo	***0·238***	***0·255***															
Sontiolo	0·015	0	0·180														
Tiolo	0·060	0	0·220	0													
Lago	−0·066	0	0·118	0	0												
Grosio	0·062	0·011	0·157	−0·054	−0·015	−0·136											
Grosotto	0·095	−0·012	***0·231***	−0·063	−0·030	−0·141	−0·037										
Prada	0·034	0	0·196	0	0	0	−0·037	−0·048									
Vione	***0·446***	***0·608***	0·140	0·382	0·507	0·232	***0·441***	***0·595***	0·431								
Mazzo	***0·651***	***0·728***	***0·643***	***0·640***	***0·685***	***0·594***	***0·683***	***0·750***	***0·657***	***0·435***							
Vervio	0·766	0·965	***0·725***	0·906	0·945	0·822	0·883	***0·930***	0·924	***0·450***	***0·082***						
Tovo	***0·648***	***0·743***	***0·689***	***0·662***	***0·703***	***0·621***	***0·704***	***0·762***	***0·677***	***0·550***	***0·195***	−0·322					
Nova	***0·639***	***0·844***	0·408	0·681	***0·780***	***0·533***	***0·719***	***0·823***	***0·723***	0·139	0·222	0·363	***0·497***				
Lovero (farm 34)	***0·877***	***1***	***0·816***	1	1	1	***0·949***	0·970	1	0·594	0·255	0	−0·017	0·629			
Lovero (rest)	−0·013	0	0·158	0	0	0	−0·081	−0·088	0	0·320	***0·620***	0·878	***0·644***	0·622	1		
Sernio	***0·809***	***1***	***0·742***	***1***	***1***	***1***	***0·932***	***0·954***	***1***	***0·764***	***0·427***	0·688	***0·344***	***0·723***	1	***1***	
Biolo	−0·066	0	0·118	0	0	0	−0·136	−0·141	0	0·232	***0·594***	0·822	***0·621***	***0·533***	1	0	***1***

Numbers in bold italics indicate those values that were significant at the 5% level after the 992 permutations in the test of Schneider *et al.* (1997).
So, Sondalo; Som, Sommacolgna; Mig, Migiondo; Son, Sontiolo; Tio, Tiolo; Lag, Lago; Gr, Grosio; Gro, Grosotto; Pra, Prada; Vio, Vione; Maz, Mazzo; Ver, Vervio; Tov, Tovo; Nov, Nova; Lo, Lovero Farm 34; Lov, Lovero (rest); Ser, Sernio.

nine 40AA individuals have additional polymorphism (Mazzo 7 and Tovo 1, 5 and 12). Of eight polymorphic sites, three are synapomorphies, while five are unique mutations.

(iii) *Allozyme study*

The following loci could be scored from well-defined zones of staining on the cellulose acetate plates: *Aco1*, *Acp1*, *Ada*, *Ak1*, *Amy1*, *Ckmm*, *Got1*, *Got2*, *Gpd1*, *Gpi1-s*, *Idh1*, *Idh2*, *Ldh1*, *Ldh2*, *Mod1* (locus for ME), *Mor1* and *Mor2* (loci for MDH), *Mpi1*, *Np1* and *Pgm1*. An additional regulatory locus was detected when screening LDH. With kidney samples, there are normally five zones of LDH activity, as expected with equal expression of two loci (*Ldh1* coding LDH-A and *Ldh2* coding LDH-B) and a tetrameric structure (Harris & Hopkinson, 1976). However, in two individuals from Mazzo and five from Tovo, only a single band (at the position of the LDH-A homotetramer) was detected, suggesting non-expression of the *Ldh2* locus. Test studies revealed only a single band in the other tissues available (liver, heart, spleen and muscle). Two loci, *Ldr1* and *Ldr2*, which regulate *Ldh2* expression in erythrocytes and liver, respectively, have been described from studies of mouse inbred strains (Lyon *et al*., 1996). The non-expression of *Ldh2* that we observed in the Mazzo and Tovo mice presumably also reflects homozygosity of an allele at a regulatory locus, but further studies are needed to establish whether the locus is *Ldr1*, *Ldr2* or another locus. Consequently, we provisionally describe it as *LdrX*, with allele *a* associated with normal *Ldh2* expression and recessive allele *b* associated with *Ldh2* non-expression.

Another recessive allele detected was the $Mod1^{n2}$ null allele already described (Fraguedakis-Tsolis *et al*., 1997). Otherwise the allele variation reflected straightforward differences in electrophoretic mobility.

Test studies showed that we could score salivary amylase (*Amy1*) in kidney and, contrary to the complexity in interpreting the saliva gland product (Lyon *et al*., 1996), we found an apparently clear and simple co-dominant system of two alleles (which we have given the provisional labels of *u* and *v*) differing in electrophoretic mobility.

In addition to *Amy1*, *LdrX* and *Mod1*, the only variable loci in Upper Valtellina were *Got2* and *Idh1*. As shown in Table 3, the distribution of alleles among localities is very striking. Mazzo and Tovo and nearby settlements (Vervio, Nova, farm 34 in Lovero), dominated by 40AA individuals and AA × Rb hybrids with high chromosome numbers ($2n = 31$ or more), differ from the remaining localities dominated by Rb races, Rb × Rb hybrids and AA × Rb hybrids with low chromosome numbers ($2n = 28$ or less). Mice from the AA-dominated localities have high frequencies of $Amy1^u$ (overall frequency for 29 mice from 5 localities: 0·93) and moderate frequencies of $Got2^b$ (0·52), $Idh1^a$ (0·36) and $LdrX^b$ (0·45, estimated). Frequencies of these alleles are much lower in the Rb-dominated localities (overall frequencies for 100 mice from 13 localities: $Amy1^u$, 0·07; $Got2^b$, 0·01; $Idh1^a$, 0·10; $LdrX^b$, 0, estimated). Therefore, there is the tendency for the alternative alleles $Amy1^v$, $Got2^a$, $Idh1^b$ and $LdrX^a$ to be at or close to fixation in the Rb-dominated localities. Given that the only other polymorphic locus (*Mod1*) is invariant in most localities, it is not surprising that average heterozygosities tend to be higher in the AA-dominated localities than the Rb-dominated localities (Table 3).

Those loci with recessive alleles (*LdrX*, *Mod1*) were excluded from the pairwise Φ_{ST} estimates and AMOVA, following Schneider *et al*. (1997). Regarding pairwise Φ_{ST} estimates, the two AA-dominated localities with reasonable sample sizes (Mazzo and Tovo) are significantly different from all Rb-dominated localities (Table 4), again consistent with the distinctiveness of the populations with 40AA and related individuals. For the AMOVA, the populations were grouped into 'Rb-dominated' and 'AA-dominated' localities, which reflects chromosomal characteristics (Table 3). This subdivision into these two groups also has a geographical and historical basis in that the AA-dominated localities are neighbouring localities within the middle of the valley that are thought to have been colonized from a distant source in the early nineteenth century (Hauffe & Searle, 1993; see also Discussion). The AMOVA revealed that 66% of total variance in allele frequencies is caused by among-group heterogeneity, 11% is due to differences among populations within groups and 23% is the result of variation within populations. Again, the distinctiveness of the Rb- and AA-dominated localities is clear.

4. Discussion

In 1993, two of us (H. C. H. and J. B. S.) hypothesized that the existing hybrid zone in Upper Valtellina can be dated from 1807, when the River Adda was blocked by a landslide opposite Sernio, causing extensive flooding from Lovero to Grosio and probably exterminating most of the mice in this area (Fig. 1; Hauffe & Searle, 1993). After the flood subsided and the villages became habitable again, this section of the valley would have been partly recolonized by Rb mice from the surrounding areas. Thus, we envisaged the 22LV race arriving from Lower Valtellina and the 26POS race from Val Poschiavo and that the 24MV race arose as a recombinant form following this recolonization (Hauffe & Searle, 1993; Piálek *et al*., 2001). We also proposed that the all-acrocentric race (40AA) was introduced

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into the valley from some distant source at that time.

On the basis of two independent morphological analyses, mtDNA sequence data and allozyme allele frequencies presented here, the 40AA race appears to be distinct from the Rb races present in Valtellina. Since hybridization can and does occur where the 40AA race meets a Rb race (Hauffe & Searle, 1993), the distinctiveness of 40AA seems to validate our proposition that this race has been introduced recently into the valley, i.e. after the 1807 flood. Nachman *et al*.'s (1994) D-loop sequences of house mice across Europe illustrate that the T or C nucleotide at site 15597 tends to be typical of a particular geographical area, but not a particular karyotype. Therefore, although a T at this site characterizes the 40AA race and a C characterizes the Rb races in Valtellina, we cannot use this information to estimate time of divergence of these races, nor pinpoint a source area for these animals; however, the nucleotide difference emphasizes that the 40AA race was probably introduced from an external source.

Overall, the allozyme heterozygosities measured here were rather low for the 40AA-dominated localities (mean 0·048, $N = 5$). Sage (1981) in a study of 56 loci and Britton-Davidian *et al*. (1989) in a study of 34 loci calculated average heterozygosities of 0·09 across European populations. For a set of samples from the British Isles and Macquarie Island, Berry & Peters (1977) found an average heterozygosity of 0·06. Our lower values may partially reflect our choice of loci (for example, we did not include esterases, which tend to be moderately to highly polymorphic; e.g. Britton-Davidian *et al*., 1989). Therefore, the allozyme heterozygosities for the 40AA populations in Upper Valtellina should not be considered abnormally low and certainly there is no clear signal of a past population bottleneck having affected the 40AA mice. To be consistent with our model that the 40AA mice arrived after the 1807 flood, this suggests that the colonization involved a reasonably large propagule of mice from a normally polymorphic source and that there has not been a substantial reduction in numbers of the 40AA mice in Upper Valtellina since then. It is envisaged that the 40AA mice arrived with agricultural products (Hauffe & Searle, 1993) and could easily have involved substantial numbers of mice. It is also possible that the allozyme heterozygosities of the 40AA populations could have been enhanced by further colonization events over the intervening years since 1807. New 40AA mice would have been more likely to integrate into and breed successfully with a pre-existing 40AA population than with a Rb population.

The allozyme variation in the Rb populations appears to be genuinely low compared with the norm for mouse populations (mean 0·010, $N = 13$; Table 3). Much of the variation that does exist in these populations seems to derive from the introduced 40AA mice (see below). There appears to be very low heterozygosity in 24UV, as shown by the H value of zero in the six villages inhabited only by this race (Table 3). This extremely low value could indicate that the 24UV is even more recently established than the other Rb races, for example, by zonal raciation, or hybridization of the 22LV and 26POS races as proposed in Piálek *et al*. (2001). Alternatively, this race may be associated with a series of severe populations bottlenecks (caused, for example, by the flood of 1807). The genetic similarity of 22LV and 26POS is probably due to introgression of these two races after 1807 following evolution in isolation (in Lower Valtellina and Val Poschiavo, respectively). The 24MV may have been born of this introgression, through zonal raciation after 1807 (Piálek *et al*., 2001) (indeed, zonal raciation is probably continuing). In addition, there is more mtDNA sequence polymorphism in the 40AA race than in the Rb races, making it reasonable to suggest that all the Rb populations are the products of at least one, possibly strong, population bottleneck. This is of interest because fixation of chromosomal variants, such as those that characterize the Rb populations, has long been considered most likely in small populations (Lande, 1979).

Only the variation at *Mod1* can reasonably be said to have originated in the Rb populations, since $Mod1^{c}$ and $Mod1^{n2}$ are only found at a high frequency in Migiondo and neighbouring villages (see Fraguedakis-Tsolis *et al*., 1997).

Only three individuals from two Rb populations had the 40AA mtDNA haplotype (Table 2). This appears to reflect a low effective migration rate of mice (in this case, females) between races, a conclusion supported by long-term ecological studies (H. C. Hauffe, unpublished data) and an analysis of hybrid fitness (Hauffe & Searle, 1998). However, the presence of $Amy1^{u}$ in Sondalo and $Idh1^{a}$ in Migiondo, suggest that introgression occasionally occurs deep into Rb populations (Table 3). Farm 34 in Lovero differs from the rest of the village both chromosomally and in allozyme composition. Sernio is also interesting as it appears to be fixed for the $Amy1^{u}$ allele characteristic of 40AA mice, despite the fact that it is a 24UV village, suggesting introgression followed by a population bottleneck.

Although 40AA is considered ancestral to the original Rb races (Britton-Davidian *et al*., 1989; Bauchau, 1990; Nachman *et al*., 1994), most previous authors accept that the races in the Rhaeto-Lombardy region evolved from each other, on the basis of shared fusions (footnote to Fig. 1; Capanna, 1982;

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Larson *et al*., 1984; Capanna *et al*., 1988; Bauchau, 1990; Capanna & Corti, 1991; Hauffe & Piálek, 1997; Piálek *et al*., 2001). This study apparently confirms this hypothesis, since the four Rb races in Valtellina are more similar to each other than to 40AA, both genetically and morphologically.

Our result that the 24UV and 26POS races are morphologically indistinguishable would appear to contradict Thorpe *et al*. (1982) who concluded from mandible and scapula measurements that the 24UV and 26POS races were clearly distinct. However, Thorpe *et al*. specify that the 24UV and 26POS individuals examined came from a sympatric population where these races were reproductively isolated: that of Migiondo (Fig. 1). The 24UV and 26POS samples used for the present morphometric studies come from various villages in Valtellina, where the races occur singly (although mice from these allopatric populations hybridize readily in the laboratory) or sympatrically (where the races hybridize naturally). Therefore, our results are not incompatible, but complementary: the fact that the 24UV and 26POS race were reproductively isolated and morphologically distinguishable in Migiondo (Thorpe *et al*. 1982), and breeding freely in populations in nearby villages (Hauffe & Searle, 1998) where they were indistinguishable (this paper) supports the hypothesis that reproductive isolation arose within Migiondo itself (see Introduction).

The conclusion from our molecular and morphological data is that all the Rb races in Upper Valtellina are genetically very similar to each other, thereby supporting the contention that, at the time the 24UV and 26POS races came into contact within Migiondo, it was the chromosomal differences between these races that most probably led to reproductive isolation, as previously proposed (Hauffe & Searle, 1992, 1993). The divergence in morphology in Migiondo revealed by Thorpe *et al*. (1982) is likely to have occurred subsequent to that.

In contrast our data suggest that the Rb and 40AA mice did differ in morphology, allozymes and mtDNA on first contact and that this difference still exists in Upper Valtellina. Thus, the strength of the genetic barrier between the Rb and standard mice in Upper Valtellina cannot be considered solely in terms of the chromosomal difference. This has major implications in terms of understanding hybrid fitness, introgression and other factors in the Upper Valtellina hybrid zone. More generally, these results suggest that not all contacts between chromosomal races of house mice can be viewed in terms of the chromosomal differences being the sole driver of subsequent divergence between the races, including reproductive isolation, and that other genetic factors have to be taken into consideration (see Searle, 1993). This is evident also for the hybrid zone in Tunisia between the Monastir and standard races that likewise differ by morphology, allozymes and mtDNA (Saïd *et al*., 1999).

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