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Mitochondrial genome variation and the origin of modern humans

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The analysis of mitochondrial DNA (mtDNA) has been a potent tool in our understanding of human evolution, owing to characteristics such as high copy number, apparent lack of recombination[1], high substitution rate[2] and maternal mode of inheritance[3]. However, almost all studies of human evolution based on mtDNA sequencing have been confined to the control region, which constitutes less than 7% of the mitochondrial genome. These studies are complicated by the extreme variation in substitution rate between sites, and the consequence of parallel mutations[4] causing difficulties in the estimation of genetic distance and making phylogenetic inferences questionable[5]. Most comprehensive studies of the human mitochondrial molecule have been carried out through restriction-fragment length polymorphism analysis[6], providing data that are ill suited to estimations of mutation rate and therefore the timing of evolutionary events. Here, to improve the information obtained from the mitochondrial molecule for studies of human evolution, we describe the global mtDNA diversity in humans based on analyses of the complete mtDNA sequence of 53 humans of diverse origins. Our mtDNA data, in comparison with those of a parallel study of the Xq13.3 region[7] in the same individuals, provide a concurrent view on human evolution with respect to the age of modern humans.

The molecular clock hypothesis postulates that DNA sequence evolution is roughly constant over time in all evolutionary lineages. We used a test[8] that compares the log likelihoods of trees reconstructed with and without the molecular clock assumption to examine the supposition that the mtDNA lineages evolve at 'clock-like' rates. The human mtDNA sequences, excluding the D-loop, have evolved at roughly constant rates ($P = 0.094$), and a relative rates test[9], using a gorilla sequence as an outgroup, demonstrates that there is also no significant difference between the evolutionary rate of human and chimpanzee mtDNAs ($P = 0.123$), excluding the D-loop. In contrast, the D-loop has not evolved at a constant rate across all human lineages ($P < 0.001$), and is consequently less suitable for dating evolutionary events. Therefore, unless specifically mentioned, we have excluded the D-loop from the analyses that follow.

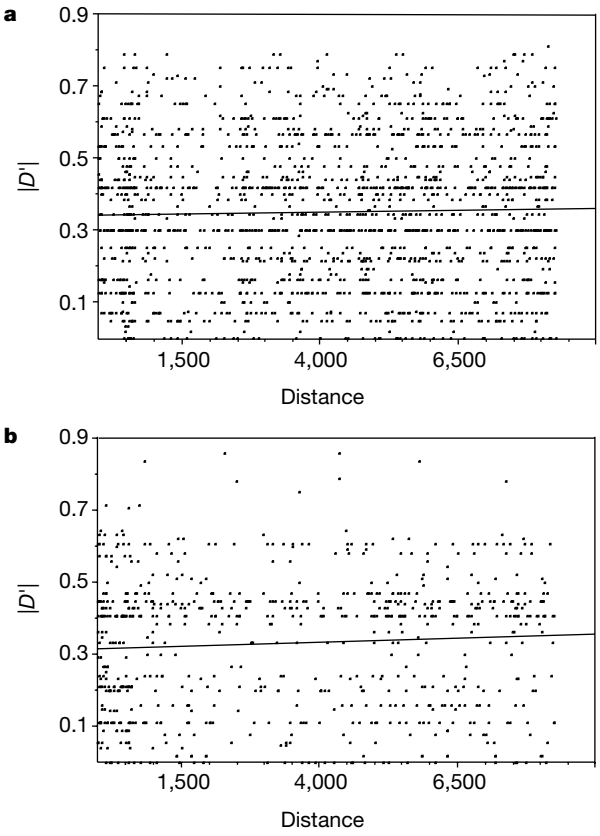

Figure 1 The relationship between linkage disequilibrium, measured by $|D'|$ versus distance between nucleotide sites for all 53 complete human mtDNA genomes. Values of ± 1.0 have been removed. **a**, Individuals of African descent ($n = 1,719$ comparisons), $R^2 = 0.001$; **b**, only non-African individuals ($n = 741$ comparisons), $R^2 = 0.005$.

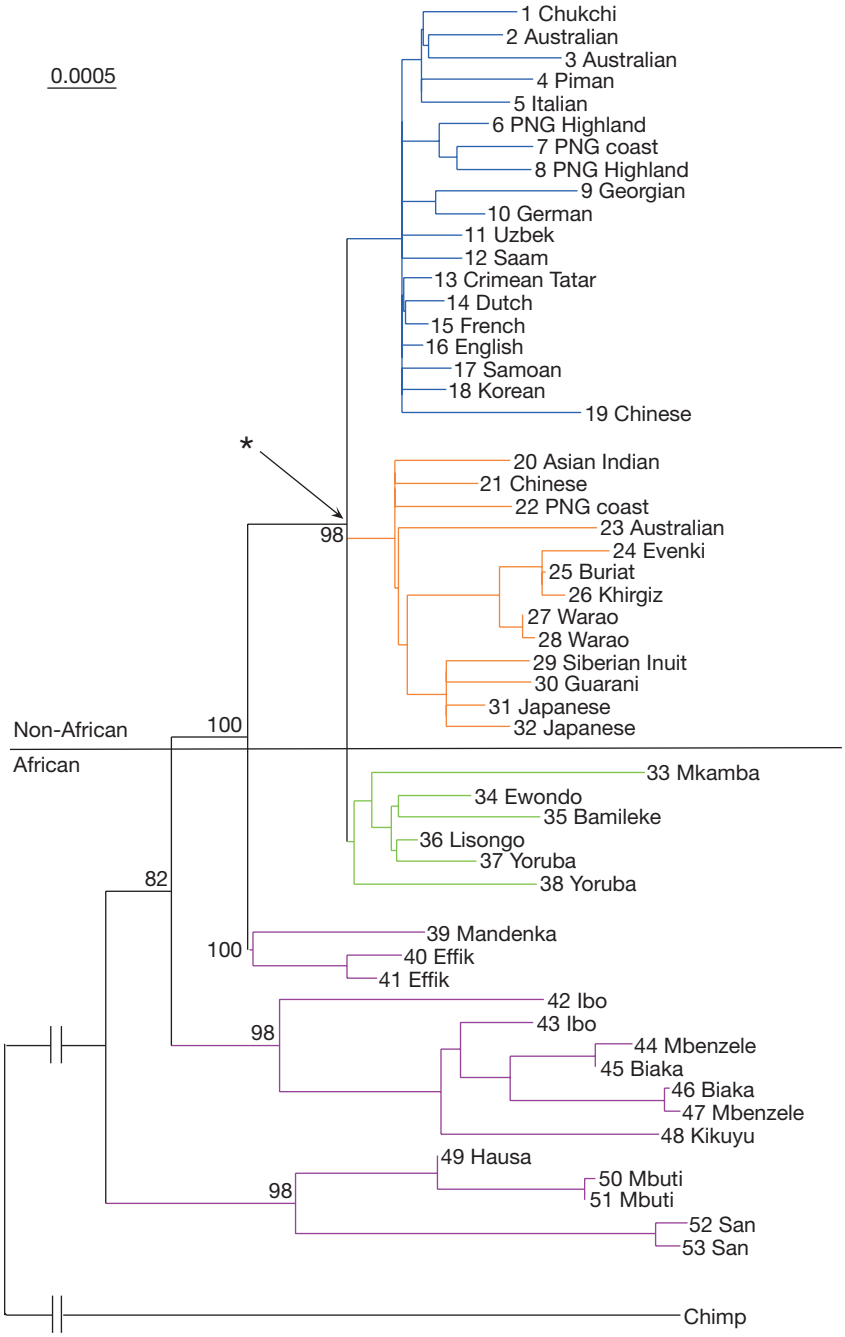

Figure 2 Neighbour-joining phylogram based on complete mtDNA genome sequences (excluding the D-loop). Data was constructed using PAUP*4.0 Beta (Sinauer Associates) and bootstrapped with 1,000 replicates (bootstrap values shown on nodes). The population origin of the individual is given at the twigs. Branches have been colour coded as in Fig. 4. Individuals of African descent are found below the dashed line and non-Africans above. The node marked with an asterisk refers to the MRCA of the youngest clade containing both African and non-African individuals.

Table 1 Summary of statistical parameters for the mtDNA

	Data set	Length	*n*	*S*	MPSD	π
Total	All humans	16,553	53	657	61.1	3.7×10^{-3}
	Non-Africans	16,555	32	358	38.5	2.3×10^{-3}
	Africans	16,556	21	367	76.7	4.6×10^{-3}
D-Loop	All humans	1,118	53	141	17.2	1.5×10^{-2}
	Non-Africans	1,118	32	103	12.8	1.1×10^{-2}
	Africans	1,121	21	77	19.7	1.8×10^{-2}
Rest	All humans	15,435	53	516	43.9	2.8×10^{-3}
	Non-Africans	15,437	32	255	25.7	1.7×10^{-3}
	Africans	15,448	21	290	57.0	3.7×10^{-3}

mtDNA data are given as entire sequences, or separated into D-loop and rest (all but the D-loop), and further separated into groups of African and non-African individuals. Length, aligned sequence length excluding gaps; *n*, number of sequences; *S*, number of segregating sites; MPSD, mean pairwise sequence difference; and π, genetic diversity.

From the mean genetic distance between all the humans and the one chimpanzee sequence (0.17 substitutions per site) and the assumption, based on palaeontological[10] and genetic[11] evidence, of a divergence time between humans and chimpanzees of 5 Myr, the mutation rate (μ) for the mitochondrial molecule, excluding the D-loop, is estimated to be 1.70×10^{-8} substitutions per site per year.

On the basis of the correlation between linkage disequilibrium and distance between sites, it has been claimed that mtDNA sequences show signs of recombination[12,13]. These analyses can be criticized for the methodology used[14], particularly for the use of a linkage disequilibrium measure (Hill and Robertson measure, r^2) that doesn't take allele frequency into account. We examined linkage

disequilibrium among all informative sites in our set of complete mtDNA genomes (including the D-loop) using a standard estimate (D') that allows all variable positions to be examined[15]. For this analysis, we studied the sequences of Africans and non-Africans separately. There is no correlation between D' and nucleotide distance between sites in the 53 sequences (African correlation coefficient, $R^2 = 1 \times 10^{-3}$; non-African $R^2 = 5 \times 10^{-3}$) (Fig. 1). We also analysed the association between r^2 and distance, and again there is no evidence of a correlation ($R^2 = 2.23 \times 10^{-6}$, and 1×10^{-3}, respectively; data not shown). Thus, it does not appear to be necessary to consider recombination as contributing to the evolution of mtDNA.

The two main hypotheses for the evolution of modern humans agree that *Homo erectus* spread from Africa around 2 Myr ago. The 'recent African origin' hypothesis[16,17] states that anatomically modern humans originated in Africa 100,000–200,000 years ago and subsequently spread to the rest of the world, replacing archaic human forms with little or no genetic mixing. The alternative, 'multi-regional' hypothesis proposes that the transformation to anatomically modern humans occurred in different parts of the world, and supports this with fossil evidence of cultural and morphological continuity between archaic and modern humans outside Africa[18]. There are, of course, variants of these two basic hypotheses that introduce additional assumptions, such as gene flow, that make the hypotheses increasingly difficult to test.

Support for a recent African origin of modern humans has been provided by a number of mtDNA studies[16,17,19,20]; however, these results have been troubled by the lack of statistical support for tree topology, especially the deep African branches[21,22]. Lacking sufficiently strong empirical data, it is impossible to confidently place the root of modern human mtDNA lineages in sub-Saharan Africa. The neighbour-joining[23] (NJ) tree constructed from our mtDNA sequences has a strongly supported basal branching pattern (Fig. 2). The three deepest branches lead exclusively to sub-Saharan mtDNAs, with the fourth branch containing both Africans and non-Africans. The deepest, statistically supported branch (NJ bootstrap = 100) provides compelling evidence of a human mtDNA origin in Africa.

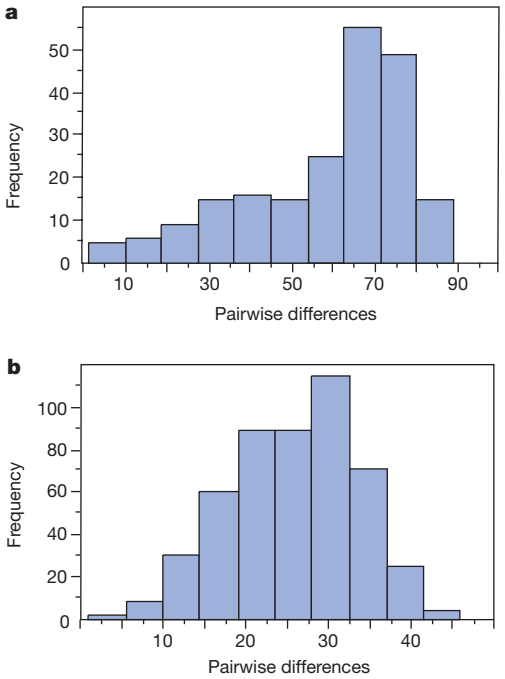

Figure 3 Mismatch distributions of pairwise nucleotide differences between mtDNA genomes (excluding the D-loop). **a**, African; **b**, non-African.

The amount of mtDNA sequence diversity (π) among Africans (3.7×10^{-3} nucleotide differences per site) is more than twice that among non-Africans (1.7×10^{-3}) (Table 1), corroborating earlier studies of the D-loop[16] and nuclear loci[24]. Also notable is the contrast between the deep branches of African mtDNAs and the 'star-like' phylogeny of non-African mtDNAs (Fig. 2). This high African diversity might result from either a considerably larger effective population size or a significantly longer genetic history. The 'star-like' phylogeny of the non-African sequences suggests a

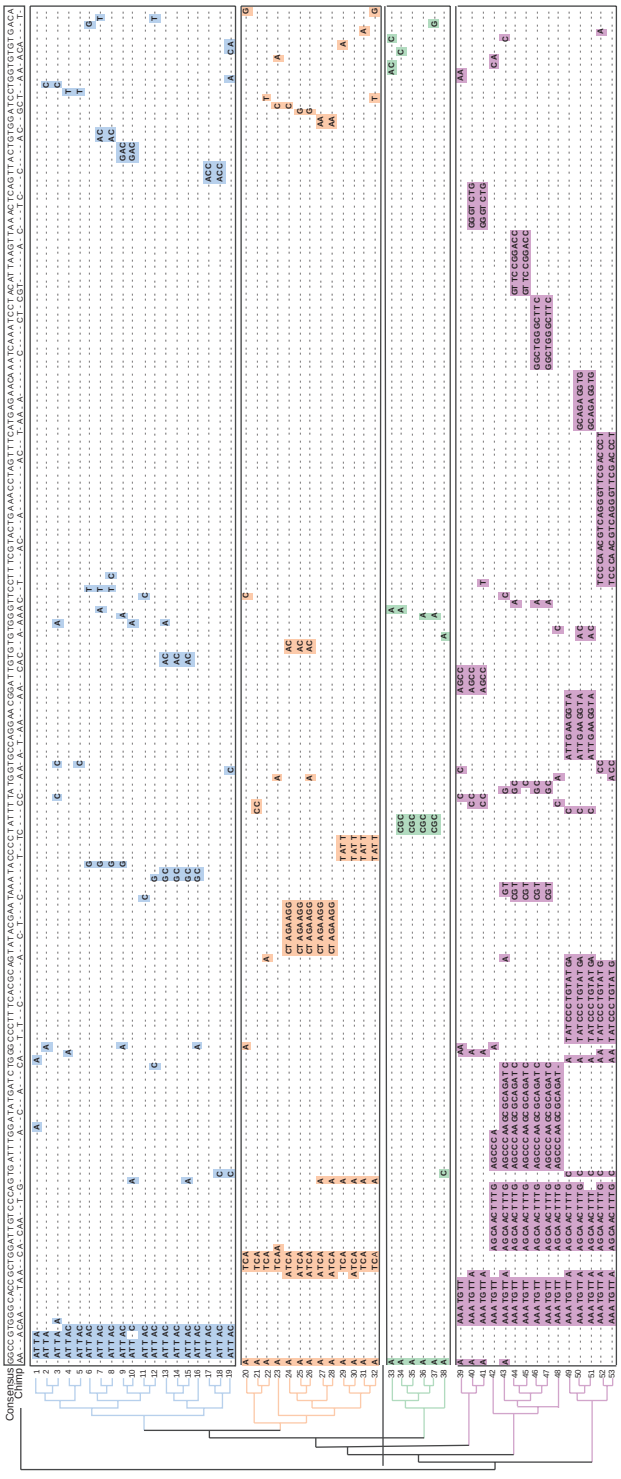

Figure 4 Data matrices showing all informative nucleotide positions, in decreasing order of frequency. Left, the whole mtDNA genome, excluding the D-loop. Right, the D-loop. The

population bottleneck, potentially associated with the colonization of Eurasia from Africa. The date of this exodus from Africa can be estimated if the departing group subsequently experienced a population expansion. The mtDNA mismatch distributions for Africans and non-Africans indicate a marked difference in population history for the two groups[25] (Fig. 3). Mitochondrial DNAs from individuals of African origin show a ragged distribution consistent with constant population size, whereas the bell-shaped distribution of the non-African comparisons clearly indicates a recent population expansion. The assumption of constant population size can be verified by tests of selective neutrality that examine the correlation between the mean pairwise sequence difference (MPSD) and the number of segregating sites (S)[26,27]. In the African group, we cannot reject this assumption (Fu and Li's $D = -1.17$ (ref. 26); Tajima's $D = -1.22$ (ref. 27)), consistent with the premise that the population has been of roughly constant size . However, it can be rejected in the non-African group ($D = -4.02$; $D = -2.28$), indicating that this group has experienced a period of population growth[25]. The

trees on the left are cladograms with the same topology and numbering of individuals as the tree in Fig. 2. Individuals of African descent are found below the dashed line and non-Africans above. The four major groups of sequences have been colour coded as in Fig. 2. Blocks denote groups of nucleotides that are identical in several sequences.

time when the expansion began was estimated ($\tau = 20.23$) to be about 1,925 generations ago[28]. Assuming a generation time of 20 yr this equates to 38,500 yr BP, a date that coincides with the onset of a period of cultural change about 35,000–40,000 years ago[29]. This involves, for example, the first appearance of regional cultural variation and the acceleration of artefactual change.

The age of the most recent common ancestor (MRCA) for mtDNA, on the basis of the maximum distance between two humans (5.82×10^{-3} substitutions per site between the Africans Mkamba and San), is estimated to be $171{,}500 \pm 50{,}000$ yr BP. We can also estimate the age of the MRCA for the youngest clade that contains both African and non-African sequences (Fig. 2, asterisk) from the mean distance of all members of that clade to their common node (8.85×10^{-4} substitutions per site) as $52{,}000 \pm 27{,}500$ yr BP. Because genetic divergence is expected to precede the divergence of populations, this date can be considered as the lower bound for an exodus from Africa.

Notably, a group of six African sequences (Fig. 4a, sequences 33–38) are genetically distant to those of other Africans, but share a common ancestor with non-Africans. These lineages represent descendants of a population that evidently gave rise to all the non-African lineages. Whether the ancestors of these six extant lineages originally came from a specific geographic region is not possible to determine, but we note that these sequences are from five populations that are now geographically unrelated.

Our study of the entire mitochondrial genome has some significant distinctions from previous studies of the D-loop. Most notably, the sequences outside of the D-loop evolve in a roughly 'clock-like' manner, enabling a more accurate measure of mutation rate, and therefore improved estimates of times to evolutionary events. Also of importance is the strong statistical support for the tree topology that has been lacking in earlier investigations. The difference between the D-loop and the rest of the molecule is visually evident in the contrast between the jumbled arrangement of polymorphic sites in the D-loop and the clear haplotypes defined by the sites in the rest of the molecule (Fig. 4).

The use of largely the same individuals in the this study, as in that of the nuclear Xq13.3 (ref. 7) region, provides a unique opportunity to compare the information gained from the two genetic systems. Because the X chromosome has an effective population size three times that of mitochondria, the MRCA of an X-chromosomal locus is expected to be three times higher. Thus, the age of the MRCA of Xq13.3 is in agreement with the mtDNA data (mtDNA: 171,500; Xq13.3: 479,000 yr BP). The results from Xq13.3 also concur with the mtDNA data with respect to the greater genetic diversity found among African individuals relative to non-Africans, but Xq13.3 shows a considerably lower difference in diversity between the two groups (mtDNA: African, 3.9×10^{-3}, non-African, 1.7×10^{-3}; Xq13.3 : 3.5×10^{-4}, 3.05×10^{-4}). Owing to its lower substitution rate, only 33 segregating sites were present in 69 sequences of 10.2 kilobases (kb) at Xq13.3, as compared with the 657 segregating sites in the mitochondrial dataset. Comparisons of the mtDNA and Xq13.3 sequences carried by specific individuals show little correlation between the two loci, as expected from the different modes of inheritance. For example, the two Warao indians showing the highest similarity in mtDNA have, in fact, two of the most divergent sequences studied at the Xq13.3 locus. Others, such as the Saami and Mandenka sequences, are closely related at Xq13.3 but have relatively high mitochondrial divergence.

Our results indicate that the field of mitochondrial population genomics will provide a rich source of genetic information for evolutionary studies. Nevertheless, mtDNA is only one locus and only reflects the genetic history of females. For a balanced view, a combination of genetic systems is required. With the human genome project reaching fruition, the ease by which such data may be generated will increase, providing us with an evermore detailed understanding of our genetic history. □

Methods

Sampling strategy and mtDNA sequences

To assess the global genetic diversity in humans, while analysing a restricted number of samples, we studied 53 individuals representing 14 of the major linguistic phyla. This sampling strategy attempts to avoid the bias inherent in selecting individuals on the basis of current world demographics, such as current population size or geographic location[5]. To provide an opportunity for comparison, we selected, where possible, the same individuals as those used by a previous study[7] for the analysis of the Xq13.3 region. All the complete mtDNA sequences are unique and vary in length from 16,558 to 16,576 base pairs (bp). From nearly 900 kb sequenced, 5 heteroplasmic sites were confidently identified. We identified a total of 657 segregating sites (141 in the D-loop; 516 outside) among these 53 individuals, of which 283 (80 in the D-loop; 203 outside) showed the same polymorphism in at least 2 individuals (Fig. 4). The pairwise sequence distances between mtDNAs, corrected for multiple substitutions[4], vary from 6.0×10^{-5} substitutions per site between two South American indians (Warao) to 6.8×10^{-3} substitutions per site between two Africans (Mbenzele pygmy and San). The average distance between mtDNA genomes is 3.8×10^{-3} substitutions per site.

PCR primers and sequencing

The primers we used for polymerase chain reaction (PCR) amplification have been described[30]. Sequencing was performed on the PCR products directly using BigDye (Applied Biosystems) chemistry. Separation of sequencing ladders was performed on the ABI 377 instrument for automated fragment analysis. We sequenced both forward and reverse strands. Sequence analysis was performed using Sequencing Analysis 3.3 (Applied Biosystems), and sequence alignment was made with Sequencher 3.1.1 (Gene Codes).

Accession numbers

The chimpanzee sequence (GenBank accession no. D38113 and the gorilla sequence (accession no. D38114) that we used as outgroups were obtained from a public mitochondrial sequence database (MITOMAP: http://www.gen.emory.edu/ mitomap.html).

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The *HIC* signalling pathway links CO_2 perception to stomatal development

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Stomatal pores on the leaf surface control both the uptake of CO_2 for photosynthesis and the loss of water during transpiration. Since the industrial revolution, decreases in stomatal numbers in parallel with increases in atmospheric CO_2 concentration have provided evidence of plant responses to changes in CO_2 levels caused by human activity[1,2]. This inverse correlation between stomatal density and CO_2 concentration also holds for fossil material from the past 400 million years[3] and has provided clues to the causes of global extinction events[4]. Here we report the identification of the *Arabidopsis* gene *HIC* (for high carbon dioxide), which encodes a negative regulator of stomatal development that responds to CO_2 concentration. This gene encodes a putative 3-keto acyl coenzyme A synthase—an enzyme involved in the synthesis of very-long-chain fatty acids[5]. Mutant *hic* plants exhibit up to a 42% increase in stomatal density in response to a doubling of CO_2. Our results identify a gene involved in the signal transduction pathway responsible for controlling stomatal numbers at elevated CO_2.

As part of a promoter trap screen aimed at isolating tissue-specific genes in *Arabidopsis thaliana*[6,7], we identified an individual plant designated *hic* in which the *β-glucuronidase* (*GUS*) reporter gene was expressed only in the guard cells (Fig. 1) and nowhere else in the plant. Southern analysis of *hic* genomic DNA using a *GUS* gene probe detected a single band, suggesting that there was only one *GUS* gene insertion in this line (data not shown).

We next investigated the phenotype of the *hic* mutant and found it to be different from previously described *Arabidopsis* stomatal development mutants[8,9]. *hic* stomata had no obvious phenotype and the plants were not wilting, suggesting that guard-cell function was not impaired. *hic* guard cells increased turgor in response to light and reduced turgor in response to the addition of the plant hormone abscisic acid in stomatal bioassays (data not shown). *hic* plants were indistinguishable from the parental ecotype when analysed by infrared thermography (data not shown); however, analysis of plants that had been grown under differing CO_2 concentrations showed that the *HIC* gene affects stomatal development.

We grew *hic* and C24, the parental ecotype, at elevated and ambient concentrations of CO_2, and measured the stomatal index and density on the leaf surface. A reduction in stomatal index and density in response to elevated CO_2 is well characterized in some species, including three ecotypes of *Arabidopsis*[1,2], and indicates that the plant has the potential for increased water-use efficiency with increasing CO_2. Table 1 shows that growth of *hic* plants under elevated CO_2 induced marked increases in both stomatal index and

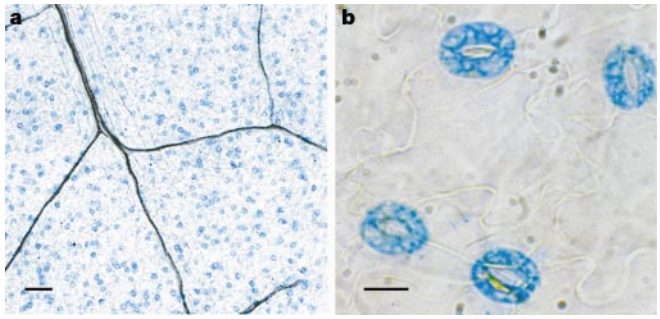

Figure 1 *GUS* expression in *hic Arabidopsis* plants is specific to guard cells. Part of a *hic* leaf histochemically stained for GUS activity shows guard-cell-specific expression at low (**a**; scale bar, 100 μm) and higher magnification (**b**; scale bar, 10 μm).

Table 1 Effect of ambient and elevated CO_2 on stomatal and epidermal cell density and stomatal index

		Stomatal density ($n\,mm^{-2}$)				Epidermal cell density ($n\,mm^{-2}$)				Stomatal index (%)			
Experiment	Line	Ambient	Elevated	Difference (*P*)	% change	Ambient	Elevated	Difference (*P*)	% change	Amnbient	Elevated	Difference (*P*)	% change
1	C24	280 (9)	233 (8)	<0.001	–17.0	1,047 (9)	902 (8)	<0.001	–13.8	21.1 (9)	20.5 (8)	NS	–3.1
	hic	231 (8)	320 (8)	<0.001	+38.0	891 (8)	902 (8)	NS	+1.2	20.8 (8)	26.0 (8)	<0.005	+25
2	C24	265 (7)	245 (9)	NS	–7.8	1,056 (7)	1,028 (9)	NS	–2.7	20.0 (7)	19.1 (9)	NS	–4.4
	hic	257 (9)	324 (10)	<0.001	+25.2	1,047 (9)	1,061 (10)	NS	+1.3	19.8 (9)	23.2 (10)	<0.001	+17.8
3	C24	324 (9)	245 (7)	<0.001	–24.5	1,322 (9)	1,058 (7)	<0.005	–20	19.6 (9)	18.7 (7)	NS	–4.7
	hic	273 (7)	387 (9)	<0.001	+41.8	1,053 (7)	1,092 (9)	NS	+3.7	20.5 (7)	26.2 (9)	<0.001	+27.6
4	C24	273 (12)	283 (12)	NS	+3.1	1,052 (12)	1,049 (12)	NS	–0.3	20.6 (12)	21.2 (12)	NS	+3.2
	hic	266 (12)	351 (12)	<0.001	+31.8	1,035 (12)	1,062 (12)	NS	+2.6	20.6 (12)	24.9 (12)	<0.001	+21.2

Data are presented for four separate experiments in the *hic* mutant and the parental ecotype (C24). The average density or stomatal index is presented for each treatment. The numbers in brackets indicate the number of individual plants in each treatment. Treatments were compared using the Mann–Whitney Rank Sum Test (SigmaStat 2.03). NS, not significant.