Parallels between island lizards suggests selection on mitochondrial DNA and morphology

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SUMMARY

Parallels between species on historically independent islands have been shown to be a powerful means of testing for the action of natural selection on morphology. Mitochondrial DNA evolution is often assumed to be selectively neutral, but this is difficult to test empirically. Here we show that parallels are present between two species of lizards on the geologically distinct but ecologically similar Lesser Antillean islands of Dominica and Basse Terre (Guadeloupe). These parallels are found not only in morphological variation but also in mitochondrial DNA variation and these are correlated with similar ecological gradients. A morphological cline in *Anolis oculatus* along the Caribbean coast of Dominica is compared to sequence variation in the cytochrome b gene, which shows exceptionally high variability. Patterns of sequence variation and morphology (especially scalation) are congruent, and are significantly correlated with moisture gradients. *Anolis marmoratus* on the adjacent island of Basse Terre (Guadeloupe) shows parallel patterns of morphological and cytochrome b variation along the Caribbean coast, and these are also significantly correlated with moisture gradients.

1. INTRODUCTION

Since the development of amplification and direct sequencing techniques, the use of mitochondrial genes (including cytochrome b) to supply information on phylogenetic relationships and divergence rates (Moritz *et al.* 1987; Meyer & Wilson 1990; Meyer *et al.* 1990; Irwin *et al.* 1991; Fajen & Breden 1992; Carr & Hughes 1993; Van Den Bussche & Baker 1993) and intraspecific population structure (Edwards & Wilson 1990; Carr & Marshall 1991; Birt-Friesen *et al.* 1992) has increased dramatically. The assumption of selective neutrality of molecular evolution (Kimura 1983; Gillespie 1991) is still widely used for its convenience in reconstructing phylogenies, although evidence to the contrary continues to accumulate. Some artificial selection experiments (MacRae & Anderson 1988; Fos *et al.* 1990; Matsuura *et al.* 1993) have indicated that mitochondrial (mt) DNA haplotype frequencies within populations are influenced by natural selection, but both their interpretation (Nigro & Prout 1990; Singh & Hale 1990; Kambhampati *et al.* 1992) and relevance is open to criticism. Although it has been known for some time that substitution rates vary within a codon of protein-coding regions of the mitochondrial genome, suggesting the operation of selective constraints (Wolfe *et al.* 1989; Palumbi & Kessing 1991), the extent to which selection influences substitutions at silent (synonymous) positions is not clear (Gillespie 1991). In general, the debate has been largely confined to theory and is seldom explicitly tested against real data at the microevolutionary level.

Studies of parallel geographic patterns between species has provided valuable evidence of natural selection on morphology at the phenotypic level (references in Endler 1986), and on allozyme frequencies (Oakeshott *et al.* 1984) at the molecular level. In particular, comparisons between closely related allopatric taxa from ecologically similar islands with independent histories has documented morphological parallels resulting from parallel selection. In the Western Canary islands, two sister species of *Chalcides* (Squamata: Scincidae) were shown to display parallel north–south changes in coloration in response to similar changes in biotype (from lush to arid) on the islands of Tenerife and Gran Canaria (Brown *et al.* 1991). Since the islands (and thus the skinks) have always been separate, a historical explanation for the parallels (requiring the same historical event to have occurred independently in both islands) is extremely unlikely and can be rejected. On the other hand, the hypothesis that similar climatic and vegetational differences within the islands exert similar selection pressures on both species cannot be rejected, and possible mechanisms by which this might occur can be tested. Parallels are thus a potentially powerful means of distinguishing between historical causes and natural selection. Here we report on a similar parallel between two species of *Anolis* (Squamata: Iguanidae) from the Lesser Antilles.

Anolis oculatus, from the ecologically heterogenous island of Dominica, has been shown to display striking geographic variation in morphology (Malhotra & Thorpe 1991*a*; Malhotra 1992). The pattern of morphological variation over the island as a whole is clinal, with individual characters showing incongruent patterns. This feature of the morphological variation is contrary to those expected from introgression following secondary contact (i.e. hybridization between temporarily isolated forms), but is consistent with primary differentiation as might be caused by natural selection (Endler 1977). Rigorous statistical hypothesis testing (Malhotra & Thorpe 1991*a*), in which observed morphological patterns were simultaneously compared with several alternative hypothesized causes (including both natural selection and neutral genetic change), strongly supported natural selection for varying environmental conditions. This correlational evidence for natural selection was further supported by manipulative field experiments (Malhotra & Thorpe 1991*b*; Thorpe & Malhotra 1992) which demonstrated extremely rapid (*ca.* 2 months) directional selection on morphotypes that had been translocated from a different habitat.

A closely related species, *Anolis marmoratus*, which is similarly morphologically diverse, occurs widely on the islands of the adjacent Guadeloupean archipelago. This study is restricted to one of these, Basse Terre, which is similar to Dominica in age, orientation, topography and climate. In both islands, the central spine of mountains and constant north-easterly Trade winds result in an uneven distribution of rainfall. The Caribbean (leeward) coasts support a more xeric type of vegetation (dry scrub woodland) compared with the Atlantic (windward) coasts, whereas rainforest flourishes in the centre of the islands. Although there is a remarkable degree of parallel ecological zonation, the islands are essentially mirror images of each other on an east–west axis because the highest mountains are in the south in Basse Terre, but in the north in Dominica.

Thus although the Caribbean coast appears relatively ecologically homogenous compared with the rest of the island, there is nevertheless a moisture gradient with a drier area of coast occurring in the rain-shadow of the highest mountain. In Dominica this is towards the north, and is apparent in both a lower annual average rainfall and a higher number of months in which there is a rainfall deficit (Lang 1967). *Anolis oculatus* also displays a striking morphological cline on the Caribbean coast (Malhotra 1992), which includes colour pattern (south Caribbean lizards are paler and lack the white marbling found in the north), scalation (they have low numbers of body scales in the south and substantially higher numbers in the north) and body proportions (they are generally more robust and stocky in the south). In an attempt to elucidate this by comparison with a 'neutral' character system, a segment of the cytochrome b gene of mitochondrial DNA was sequenced.

2. METHODS

(*a*) *Morphological parallels between the species*

Morphological distance matrices representing multivariate generalizations of three character systems (body proportions, scalation and colour pattern) were derived from data collected from 33 localities in Dominica and 25 localities in Basse Terre. Sample sizes equalled 10 specimens in most cases but were lower (minimum = 4) in a few montane localities where lizard population densities are low. These 'observed pattern' matrices were simultaneously compared with several 'hypothesized pattern' matrices representing environmental variation (based on altitude, rainfall and vegetation data (Malhotra & Thorpe 1991*b*)) and also geographical proximity (to minimize spatial effects). Significance of the matrix correlations were obtained using a randomization procedure (partial Mantel test (Thorpe 1991), 2000 randomizations).

(*b*) *Morphological variation along the Caribbean coast*

A 22 km transect, covering the area of morphological change on the Caribbean coast of Dominica was studied. Lizards were sampled at frequent intervals (11 localities, with a sample size of between 8 and 10 specimens) along the transect, and several morphological characters were measured. These included snout–vent length, head length, snout width, tail crest depth (body proportions), number of scales at mid body, degree of scale enlargement (scalation), degree of spotting on the head, width of black patches on the trunk, number of small, medium and large white spots on the trunk, dorsal hue, number of vertical and horizontal white bands on the trunk and extent of reddish colour on head and trunk (colour pattern). Details of how these characters were measured are given in Malhotra (1992). Variation in these characters was summarized by a canonical variate analysis (thus removing the correlational effects of growth) or principal component analysis (for colour pattern, as the matrix is heteroscedastic). Scores on the first ordination axis were then plotted against distance along the transect for each character system, and these were also correlated with environmental variables (see below).

The same procedure was followed for *Anolis marmoratus* in Basse Terre, with minor differences: fewer localities were sampled (six), and the characters studied were snout–vent length, head width, toe length, leg length, (body proportions), number of scales at mid body, number of fourth-toe lamellae, number of supralabials (scalation), black patch width, number of small, medium and large white spots, dorsal hue, ventral hue and eyeskin hue (colour pattern).

(*c*) *Mitochondrial DNA sequence variation*

Patterns of sequence variation in a 267 base pair (b.p.) fragment of the mitochondrial cytochrome b gene (corresponding to sites 14851–15117 in the human sequence) were compared with the morphological clines. Tissue samples for cytochrome b sequencing were obtained by non-destructive sampling of the tip of the tail (which is readily shed and regenerated by the lizards). Whole genomic DNA was extracted, the required section was amplified by the polymerase chain reaction (PCR), using truncated versions of the 'universal primers' for cytochrome b (Kocher *et al.* 1989) and sequenced by the double-stranded dideoxy chain termination method following standard published protocols. Each sequence was verified by sequencing from both primers and from different PCR amplification products to eliminate the possibility of amplification errors. Sequence differences were given a binary score such that 1 represented identity with the 'master sequence' and 0 represented non-identity. In most cases there were only two alternative bases represented, but a few positions required more than one binary character to represent the variation in this way. Overall divergence of different sequences could then be summarized by principal component analysis. This procedure does not distinguish between transversions and transitions, but at this level of differentiation transversions are not likely to have saturated (Brown 1983). Populations were represented by two or three individuals, and the many

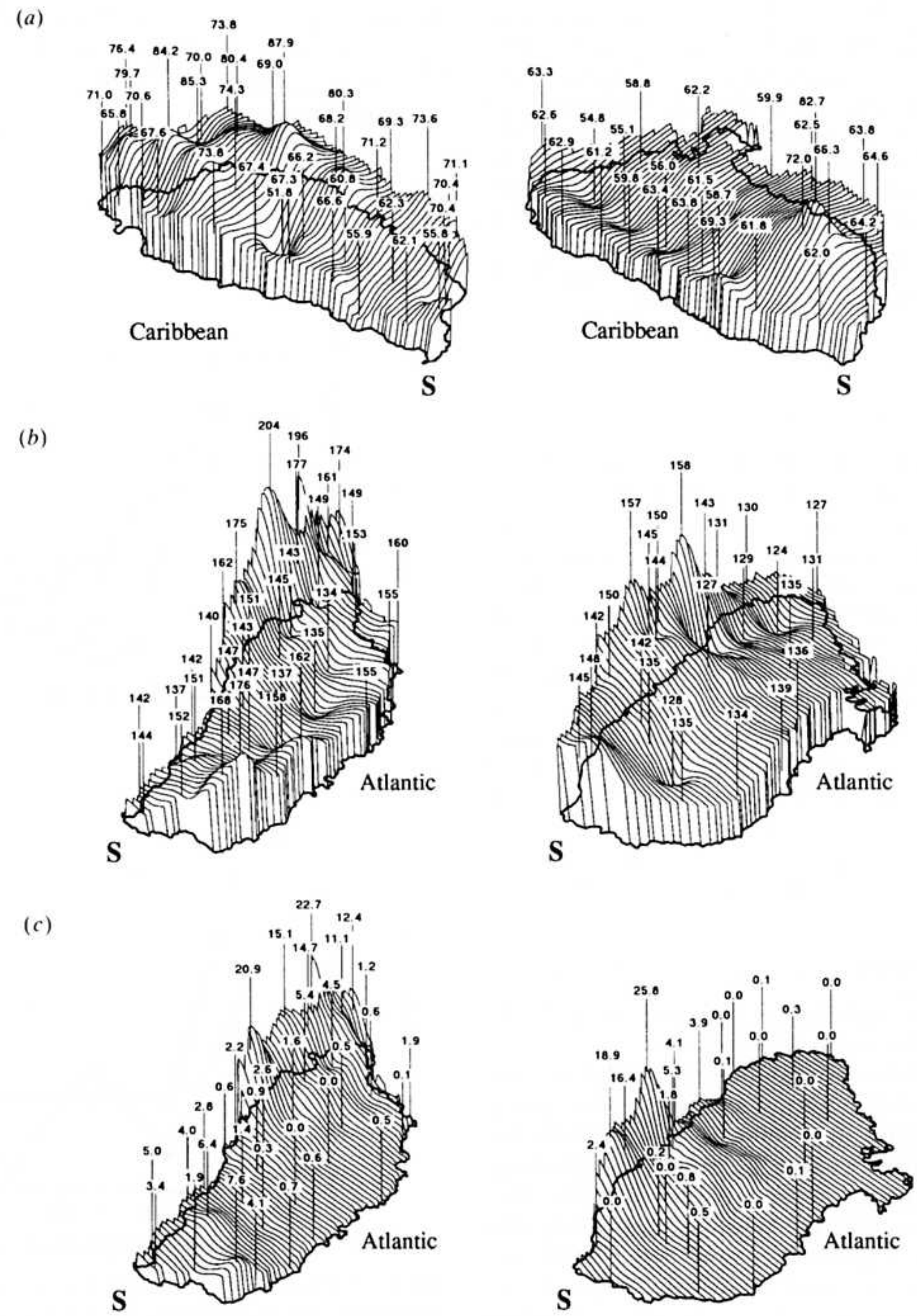

Figure 1. Surface contours of representative morphological traits from three character systems showing parallels between *Anolis oculatus* from Dominica (left), and *Anolis marmoratus*, Basse Terre (right). The height of the surface above the base map represents the value of the character in question at that point. Population means (33 in Dominica, 25 in Basse Terre), from which contours are extrapolated, are posted for each character. (*a*) Snout–vent length. (*b*) Number of scales at mid-body. (*c*) Number of light spots covering more than 25 scales. Both islands, which are approximately 40 km long, have mountainous spines (reaching nearly 1500 m) which create wet and rainforested interiors. The vegetation of the moist Atlantic (windward) coasts are dominated by salt-tolerant species. Although the Caribbean coasts are relatively dry, moisture gradients occur because the highest mountains create drier areas to their lee. As these are in the south of Basse Terre, but the north of Dominica, the islands are ecological mirror images of each other. Variation in the above morphological characters corresponds to this ecological zonation, with the largest snout–vent lengths at the highest altitudes and the highest number of scales and light spots in the driest part of the islands. In both species, body size is significantly correlated with both altitude ($|r| = 0.15$ and $|r| = 0.39$, respectively) and rainfall ($|r| = 0.13$ and $|r| = 0.39$). The number of body scales in both species is significantly correlated with rainfall ($|r| = 0.26$ and $|r| = -0.06$) and altitude ($|r| = -0.19$ and $|r| = 0.06$), and the number of spots with occurrence of dry scrub woodland ($|r| = 0.25$ and $|r| = 0.13$). All correlations are significant at $p < 0.0005$, except the number of body scales in *A. marmoratus*, which is significant at $p < 0.05$.

variable sites resulted in few sequences being exactly identical. The principal component scores were therefore calculated separately for individuals, and averaged to give a population score.

(*d*) *Environmental variation*

Information on ecological variation along the Caribbean coast of Dominica was required, on a finer scale than that generally available from the literature, to test ecogenetic hypotheses. Temperature, relative humidity and amount of light penetrating the canopy were measured using hand-held meters on several days in June 1992 and April 1993, and averaged. Rainfall data (average annual rainfall and the number of months of rainfall deficit) was obtained from the literature (Lang 1967). The character systems were compared with these five ecological gradients using a partial correlation analysis which included geographical distance along the transect. Rainfall data (average annual rainfall) for Basse Terre was obtained from Météo-France.

3. RESULTS

(*a*) *Morphological parallels between species*

Similar relationships between morphology and environmental variation were found in both species. Generalized body proportions are significantly correlated with both altitude ($|r| = 0.16$ in *A. oculatus*; $|r| = 0.28$ in *A. marmoratus*) and rainfall ($|r| = 0.13$; $|r| = 0.17$), generalized scalation with rainfall alone ($|r| = 0.14$; $|r| = 0.15$) and generalized colour pattern with vegetation type ($|r| = 0.13$; $|r| = 0.12$). All the correlations are significant at $p < 0.0005$. These parallels are visually represented by three-dimensional contour diagrams of representative characters from each of the three character systems (figure 1) reflecting these same relationships. This strongly suggests that natural selection is responsible and that it is acting in a similar way on both species (Brown *et al.* 1991).

(*b*) *West Coast Transect: congruent patterns of change*

The obvious morphological divergence between the south and the north of the Caribbean coast is well illustrated by the scores on the first ordinal axis for each character system plotted against distance along the transect (figure 2). Sequence differences in the cytochrome b gene also show geographical patterning, with a stepped cline present towards the south. This cline is congruent with the morphological clines, and especially so with generalized scalation (figure 2*a*). Surprisingly high levels of variability are present in cytochrome b sequences (12.7% base-pair differences among all sequences, with 7.8% amino-acid differences). When compared to ecological variation along the transect, the patterns of variation in both scalation and cytochrome b are significantly partially correlated with relative humidity (partial $r = -0.86$ and -0.78 respectively; Bonferroni corrected $p < 0.05$).

(*c*) *Parallels in* Anolis marmoratus

Many morphological characters in *Anolis marmoratus* show a similar cline along the Caribbean coast of Basse Terre (figure 1*b*, *c*). The cytochrome b sequence of this species was also examined in relation to morphological and ecological clines. Although sequence variability is somewhat lower in *A. marmoratus* (overall 7.8%), a parallel stepped-clinal change from north to south is also present (figure 2*b*), which is congruent with the cline in scalation characters. Most telling, however, is the fact that both character systems are also significantly partially correlated with the moisture gradient (partial $r = 0.70$, Bonferroni corrected $p < 0.01$, cytochrome b; partial $r = 0.38$, Bonferroni corrected $p < 0.05$, scalation).

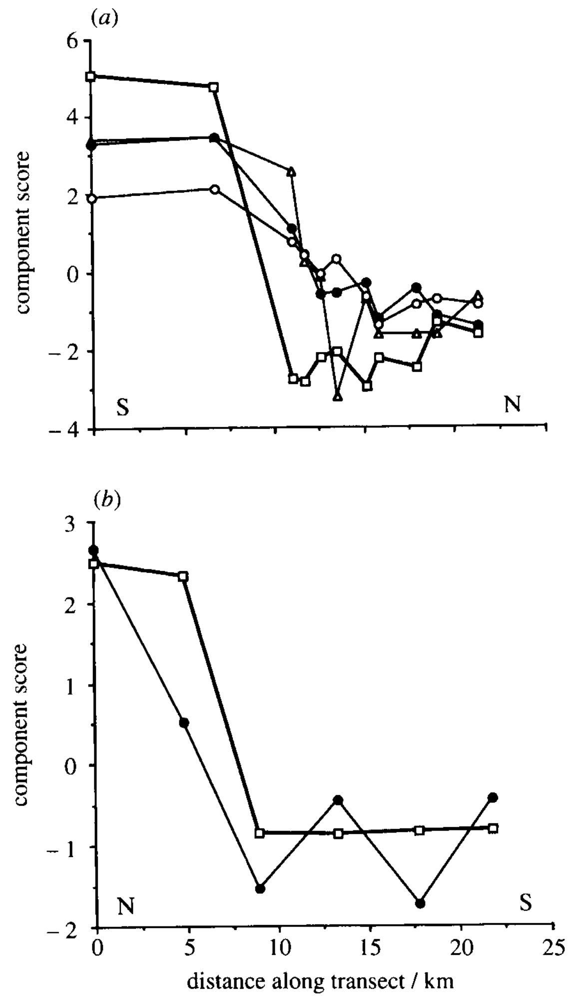

Figure 2. Parallels between patterns of variation in morphological character systems and cytochrome b along a 22 km section of the Caribbean coasts of Dominica and Basse Terre. The transects are plotted from wet to dry in both cases (thus reversing their geographical orientation). The vertical axis represents the first principal component scores (or canonical variate scores for body proportions and scalation) of the generalized character system. Cytochrome b (open squares), scalation (filled circles), body proportions (open triangles). Only those morphological character systems that show significant correlations with cytochrome b are shown. (*a*) *Anolis oculatus*, Dominica. The percentage of the total variation represented for each character system is 40%, 87%, 46% and 49% for the above systems respectively. All the characters systems are significantly correlated with each other ($p < 0.01$). The pattern of cytochrome b shows the highest congruence with scalation ($r = 0.84$; $p < 0.001$). (*b*) *Anolis marmoratus*, Basse Terre. The components represent 60% and 85% of the variation in cytochrome b and scalation respectively. Note that cytochrome b in this species also shows a stepped cline which is congruent with the pattern of variation in scalation characters ($p < 0.05$). The sequence data are available from the authors on request.

4. DISCUSSION

It is doubtful whether the conclusion that the natural selection hypothesis could not be rejected would be very contentious, if this paper concerned had itself with morphology alone. This is especially true considering that the direction of change in morphological change in both species is predictable along the environmental gradient (and see references given in the introduction for additional lines of evidence in its favour). The obvious corollary here (that this also applies to the cytochrome b sequence) is, however, likely to be contentious, hence further discussion of the alternatives is warranted. There are several unusual features of the patterns of mtDNA variation described here among which are the exceptionally high sequence divergence within both these species (but especially *A. oculatus*), and the steepness of the observed cline.

(*a*) *Historical cause* (*neutral molecular evolution*)

This would explain the patterns as a result of secondary contact and hybridization between forms that had been separated by a vicariant event (Wilson *et al.* 1985; Avise *et al.* 1987). The high degree of variation would then be related to the time since the vicariant event. However, the evidence is not consistent with this. Assuming a molecular clock applies, and a commonly accepted value for rate of evolution in vertebrate cytochrome b (2.5% per million years) is used (Kocher & White 1989), the expected time of divergence between these geographically close, intraspecific populations would be of the order of 3–5 million years. Little information is available regarding the date of arrival of the ancestral anoles on these islands, but the island chain itself is thought to have originated in the late Miocene (Martin-Kaye 1969) with extensive volcanism continuing into the Pliocene (no Miocene or pre-Miocene rocks are known to exist in Dominica). This relatively short evolutionary timescale would require that cytochrome b evolution in these species would have been unusually fast (especially since this is a poikilothermic animal hence is likely to have a slower rate (Martin & Palumbi 1993*a*). Moreover, there is no plausible vicariant event in either island. However, this interpretation could not be ruled out had this study been done on *Anolis oculatus* alone. The additional evidence from parallels between the two species (which are from islands with independent histories) would require similar vicariant events, occurring independently on both islands, to be hypothesized. This is non-parsimonious and so unlikely that it can be rejected.

(*b*) *Natural selection* (*non-neutral molecular evolution*)

As mentioned earlier, our case for selection rests on logically strong grounds. Neither the steepness of the cline or the high degree of sequence variation are inconsistent with natural selection (Endler 1977, 1982). However, the mechanism by which selection might produce such a pattern of sequence variation is unknown. Although most of the substitutions are silent, amino acid replacements occur in *A. oculatus* as a result of substitutions in second (and sometimes first and third) positions. The areas of the protein that relate to the sequenced fragment include a short portion of both reaction centres (Howell 1989; Irwin *et al.* 1991), but the consequences of the amino acid substitutions in relation to function is not clear. Even for silent substitutions there may be grounds for suggesting that selection, albeit weak, is involved (Gillespie 1991).

We are not denying that a wealth of data on cytochrome b evolution exists which is in accord with neutral assumptions (e.g. Martin & Palumbi 1993*b*), but we emphasize that if we had dealt solely with morphological traits, the evidence presented here would be accepted as indicating natural selection (Brown *et al.* 1991). Many puzzling aspects remain (such as why silent substitutions should show any kind of parallel geographic patterning in the absence of a historical cause), but the results are sufficiently striking to demand an explanation outside the neutral theory of molecular evolution, and at the very least argue for a more cautious approach to interpretations of short sections of sequence from a single protein-coding gene that depend on assuming neutral evolution. This includes both phylogeny reconstructions and studies of population-level phenomena (Avise *et al.* 1987; Neigel *et al.* 1991). Whether other parts of the mitochondrial genome (both coding and non-coding) in these particular species show similar patterns, and the manner in which they relate to nuclear differentiation are the subject of ongoing research and should help to further elucidate the processes at work.

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