Relating geographic pattern to phylogenetic process

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SUMMARY

Observed patterns of within-species geographic variation may reflect phylogenetic history or ecogenetic adaptation, and it is frequently desirable to evaluate their relative contributions. Phylogenetic information can be recovered from relatively neutrally evolving molecular markers, but to what extent are they useful in understanding the causes of observed geographic patterns within species? In the lacertid lizard *Gallotia galloti* (Western Canary islands), it allows the colonization sequence to be hypothesized, giving a new perspective on the causes of morphological differentiation between island populations. In South-East Asian pit-vipers, venom evolution can be evaluated in relation to biogeographic processes and current ecology. At a finer geographic scale, alternative historical and geological hypotheses can be tested to explain within-island microgeographic variation in *G. galloti* in Tenerife. Similar work on *Anolis oculatus* within the island of Dominica, however, raises questions concerning some of the assumptions behind a molecular phylogenetic approach.

1. INTRODUCTION

Studies of geographic variation have provided many insights into evolutionary processes. These include the importance of natural selection for locally varying conditions and the role of allopatric differentiation in the divergence of lineages. Until recently, most studies on geographic variation were concerned with morphology, but this may be influenced by both processes and their relative importance is difficult to assess. Various quantitative tests have been evaluated for this purpose, e.g. patterns of congruence between character sets (Thorpe 1991), the pattern of anagenesis in phylogenetic trees (Thorpe 1991), and tests of association between observed morphological patterns and those expected from various hypothesized causes such as vicariance or ecological adaptation (Brown & Thorpe 1991*a*, *b*; Brown *et al.* 1991; Dow *et al.* 1987; Malhotra & Thorpe 1991*a*; Sokal *et al.* 1991; Thorpe 1991; Thorpe & Brown 1991; Thorpe & Baez 1993). These methods have been discussed in detail previously (Thorpe *et al.* 1991, 1994*a*) and will not be described here. Although they can be useful, it was not until the development and increasing accessibility of molecular techniques (e.g. polymerase chain reaction (PCR)-based sequencing of the fast-evolving mitochondrial genome) that intraspecific molecular phylogenies could be readily constructed and it became possible to assess the causes of geographic variation against an independently derived hypothesis of phylogeny.

Recent years have seen the publication of phylogeographic studies on diverse organisms, e.g. swallowtail butterflies (Sperling & Harrison 1994), sparrows (Zink & Dittmann 1994), whitetailed deer (Ellsworth *et al.* 1994), salamanders (Moritz *et al.* 1992) and African elephants (Georgiadis *et al.* 1994), with further examples given in a recent review by Avise (1994). A common feature of these studies is that the molecular phylogenies obtained (from restriction fragment length polymorphisms, or, more recently, from sequence information) are interpreted in terms of various biogeographical and historical scenarios. Many studies go no further than this. However, a molecular phylogeny can also be used to quantitatively test observed patterns of geographic variation. We illustrate this with four examples.

2. CANARY ISLAND LIZARDS: COLONIZATION SEQUENCE AND THE CORRELATES OF EVOLUTION

The western Canary island lacertid, *Gallotia galloti*, is a medium-sized diurnal herbivorous lizard found on the ecologically heterogeneous islands of Tenerife, La Palma, El Hierro and La Gomera. Many morphological characters from the scalation, colour pattern, and body dimensions vary substantially between islands as well as within Tenerife. For example, the mature males on El Hierro tend to be small and blackish with blue spots on the legs and relatively robust heads, whereas in north Tenerife they tend to be large with bright yellow dorsal cross bars, blue cheeks and relatively small heads.

The western Canary Islands are known to have erupted from the sea independently, are separated by deep channels and are not thought ever to have been connected to each other or with the African mainland (references in Thorpe *et al.* 1994*b*). Thus the lizard's present distribution can be interpreted in terms of over-water colonization, but the colonization sequence cannot be recovered from a phylogenetic tree based on morphology because this may be compromised by ecogenesis. A Fitch–Margoliash tree based on combined mtDNA sequence information available (over 1005 base pairs (b.p.) from the cytochrome *b*, cytochrome oxidase and 12*S* rRNA genes) indicates that there is a distinct intraspecific phylogeny with northern (comprising populations from Tenerife and

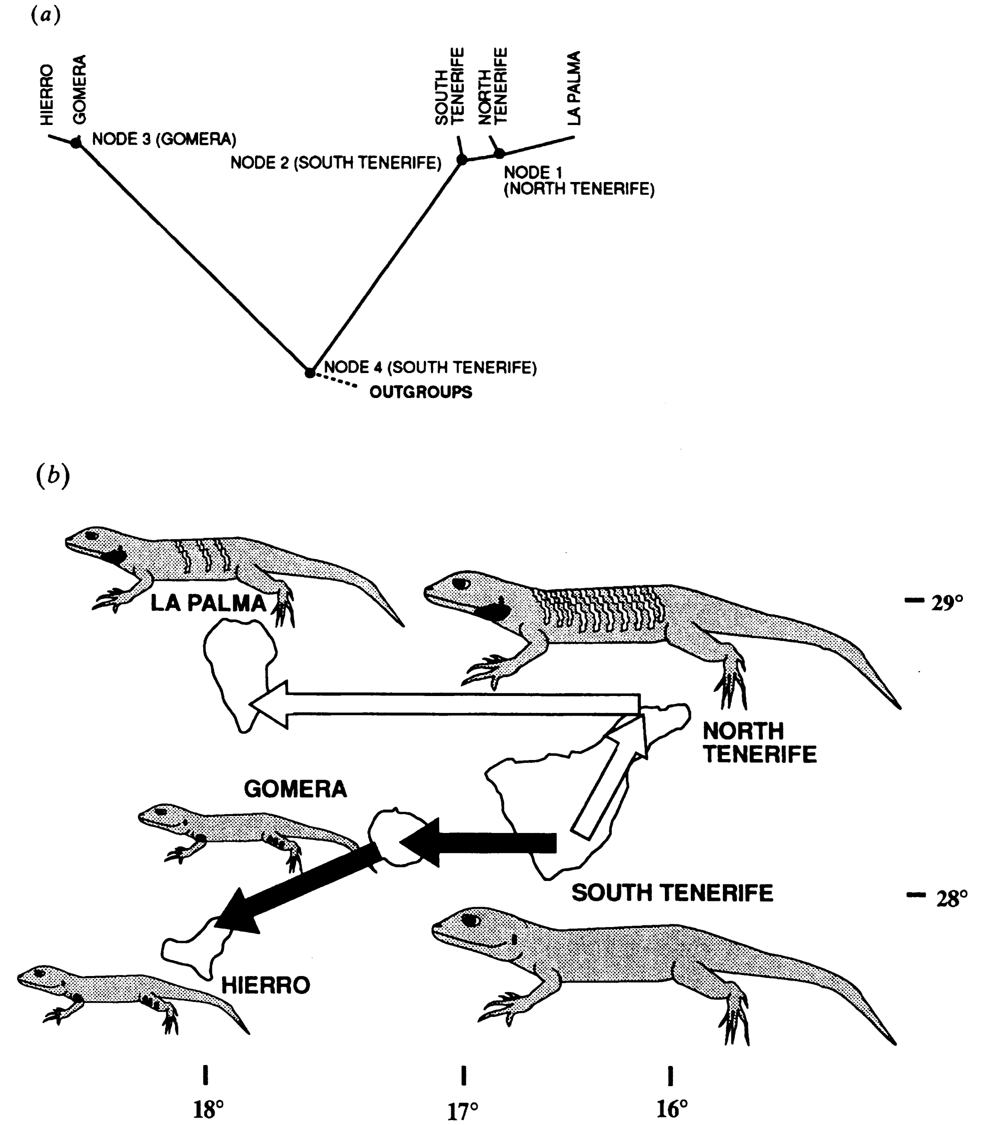

Figure 1. (*a*) Fitch–Margoliash tree (without the assumption of a molecular clock) derived from 1005 b.p. of mitochondrial gene sequence data, showing relationships of the western Canary Island lacertid *Gallotia galloti*. (*b*) Schematic representation of evolution in *G. galloti*. The arrows indicate the rigorously hypothesized colonization sequence of the two lineages (northern lineage, empty arrows; southern lineage, shaded arrows). Blue spots on the legs are thought to be for conspecific signalling and are present in the southern lineage only (phylogenesis), whereas sexually mature males are large in biodiverse islands (Tenerife), smaller in more depauperate areas (La Palma) and smallest in the most depauperate islands (Gomera, Hierro), irrespective of phylogenetic lineage. Similarly, lizards from wet, lush areas (N. Tenerife and La Palma) have disruptive yellow bars, blue cheeks and gracile heads (ecogenesis), although phylogenetic lineage has an effect in the number of bars present.

La Palma) and southern (populations from Gomera and Hierro) lineages. The colonization sequence can then be deduced from this tree by following the method of Thorpe *et al.* (1994*a*) with the use of tree topology plus branch length, or topology plus geography. This gives us an origin for the species in south Tenerife, dispersal to north Tenerife, then a westward colonization to La Palma. In the south, an independent colonization event resulted in the colonization of Gomera and subsequently of Hierro. Not only is the sequence compatible with the geological history (Tenerife being the oldest island and the islands to the west progressively younger), but if one uses a molecular clock assumption (Brown *et al.* 1982; Kocher *et al.* 1989; Wilson *et al.* 1985) then so is the timing (Thorpe *et al.* 1993*a*, *b*, 1994*a*, *b*), with the geological origin of each island preceding the time of arrival of the lizards on it.

Does the morphological variation observed in this species reflect this dispersal history, or is it more influenced by marked differences in environmental conditions among the islands? Because the phylogenetic tree was constructed from a data set independent of morphology, it can be used without circularity to test these alternative hypotheses. A partial Mantel matrix correlation test was performed (Thorpe 1991; Thorpe *et al.* 1994*a*, and references therein). This method uses a randomization procedure to estimate probabilities for the correlation between matrices, standard significance tables being invalidated by the non-independence of matrix elements. Here we use an extension of the simple two-matrix Mantel test, a partial Mantel test that is based on a multiple regression (B. F. J. Manly, personal communication) and allows up to eight independent matrices to be compared with the dependent matrix simultaneously, thus largely removing the inter-correlation problems associated with multiple hypothesis tests. Each morphological character (represented by a similarity matrix between individuals) was tested simultaneously against the phylogeny (represented by a patristic distance matrix derived from the above Fitch–Margoliash tree) and two natural selection hypotheses represented by matrices of environmental variation (derived from data on environmental richness or biodiversity and climate). Although many characters reflect both the phylogeny and environment, there are a few characters that are predominantly influenced by a single factor. For example, blue spots on legs can be shown to be closely associated with phylogeny, irrespective of ecology, whereas body size appears to be primarily related to environmental richness, with lizards of both lineages being smaller on the depauperate western islands. Other characters are associated mainly with wet, lush climates, e.g. lizards from north Tenerife and La Palma have gracile heads, blue cheeks and yellow dorsal bars (figure 1*b*).

3. THE CAUSES OF VENOM VARIATION IN THE MALAYAN PIT-VIPER

A similar approach can be taken for other types of geographical variation; for example, the study of venom variation in venomous snakes. It has been well documented (Warrell 1989) that clinical symptoms produced by snakebite can vary quite markedly between different populations of the same species, yet the causes of venom variation are poorly understood. Do they reflect neutral molecular change, or adaptation to local conditions such as available prey types? This study examined venom variation in the Malayan pit-viper, *Calloselasma rhodostoma*, which occurs in lowland forests and plantations in Java, northern West Malaysia, Thailand, Cambodia, Laos and southern Vietnam (figure 2), and is the leading cause of venomous bites in many areas (Viravan *et al.* 1992). Venom and tissue biopsies were obtained from 131 Malayan pit-vipers caught in the field, from 36 localities in Java, Malaysia, Thailand and Vietnam. Faecal material was also collected to build up a picture of the species' prey, supplemented by the identification of some 200 prey items in the gastrointestinal tracts of museum and road-killed specimens. The diet shows distinct ontogenetic and geographic variation (figure 2), and minor sexual differences. An 800 b.p. section of the mitochondrial cytochrome *b* gene was amplified by PCR and cut using seven 5- and 4-cut restriction enzymes. The patristic distances between localities on a Fitch–Margoliash tree constructed from these data represent their phylogenetic relationships. Individual venom samples were isoelectrically focused (IEF) across a high-resolution polyacrylamide gel (pH 3.5–9.5) and the protein banding patterns were compared. Ontogenetic differences were found, with the most marked changes occurring at sexual maturity, and variation was also obvious among venom samples collected from different geographic areas. IEF profiles from adult snakes (snout–vent length (SVL) exceeding 400 mm) were then used to construct distance matrices representing similarity/dissimilarity of venom composition among localities. Thirteen bands were found to vary between groups (the criteria being that variable bands are those present in at least two groups and absent from no less than two). In addition, two bands were found to be present only in females, and were excluded from further analysis. Matrices representing the overall venom composition (combining all bands) were constructed, in addition to matrices representing variation in individual bands. Similarity matrices were also produced to represent diet (the mean proportion of amphibians, reptiles and mammals consumed by adults of each population), phylogenetic relationships (patristic distances) and geographic proximity. A partial Mantel test with the overall venom composition of adults as the dependent variable, and diet, geographic distance and patristic distance as independent variables, found diet alone to be significantly partly correlated ($P < 0.001$ after Bonferroni correction). When individual variable bands are tested they are

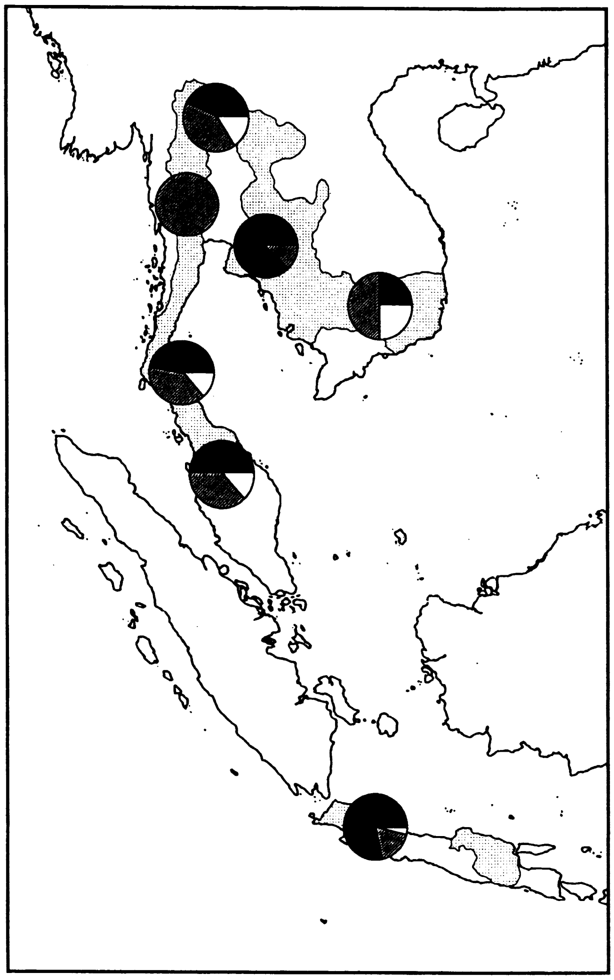

Figure 2. Distribution and adult diet composition of *Calloselasma rhodostoma* (Malayan pit-viper). The shaded area represents its distribution, and the superimposed pie charts show the relative proportion of amphibians (white), reptiles (diagonal stripes) and endotherms (black) in the diet of adult snakes from different areas.

seen to vary in their association with different factors, e.g. some bands are associated with geographic distance, probably reflecting the opportunity for genetic exchange between spatially close localities. Almost half of the bands are significantly associated with average adult diet at each locality. Only one band is solely associated with phylogenetic relationships, which calls into question the use of venom as a taxonomic tool (Tu & Adams 1968; Chen *et al.* 1984). Although the variable proteins have not yet been identified, it has been shown that there are also geographic differences in the necrotic and toxic effects of the venom (Daltry *et al.* 1995).

Different prey taxa vary in their susceptibility to venom (Minton & Minton 1969) and it appears that natural selection has caused snake populations to produce venom locally adapted to their particular diet. Studies of captive-bred *C. rhodostoma* indicate that this venom–prey association is inherited rather than environmentally induced (Daltry *et al.* 1985). Intraspecific variation in venom has important implications for snakebite therapy (Anderson *et al.* 1993; Wüster *et al.* 1992) and although it would be helpful to be able to predict the distribution of clinically significant proteins on the basis of phylogenetic relationships or geographic distribution, this study suggests that painstaking biochemical analysis may be the only solution in some cases.

4. WITHIN-ISLAND GEOGRAPHIC VARIATION IN LIZARDS

So far the cases discussed have been on a relatively large geographical scale, where we might expect to be able to find enough genetic variation between populations to be amenable to analysis. However, as long as genetic variation is detectable, this approach can also be used to study fine-level processes, such as those occurring within islands. Two examples are given.

(*a*) **Gallotia galloti** ***within Tenerife***

The island of Tenerife has a geologically complex, but well documented history. It has existed in its present form for only a comparatively short period, although the oldest rocks found date as far back as 15.7 Ma ago (Ancochea *et al.* 1990). On the basis of the presence of these ancient basaltic rocks, three possible precursor islands have been identified (figure 3*a*). These are Anaga, Teno and the Roque del Conde (also known as Adeje). Later extensive volcanic activity, which continued until around 200000 years ago, created the Las Cañadas edifice between these precursors, eventually joining them. It is possible that Teno and Adeje might originally have been a single island, with intervening rocks having been overlain by later volcanic deposits. Fossils found on Tenerife date the arrival of lacertids on the island to at least 5 Ma ago, well before the eruption of Teide; hence they must have been present on one or more of the precursor islands.

However, morphological studies do not help us to decide whether more than one lineage is present on the island. Tenerife today is an environmentally heterogeneous island, with the huge 3718 m cone of Teide creating a semipermanent layer of thick cloud at mid-altitude to the north. This results in a dramatic ecotone between lush forests on the north-facing slopes and barren semi-desert on the southern slopes. Previous studies of within-island morphological variation in Tenerife *G. galloti* have revealed a strong association with these environmental patterns. In particular, it was proposed that the north–south variation in colour pattern was due to an evolutionary trade-off between sexual selection for bright colours and natural selection for crypsis, mediated by both visual characteristics and thermal considerations imposed by the different habitat types (Thorpe & Brown 1989, 1991). An independent assessment of the phylogeny is required to test the various scenarios for the origin and spread of the species on Tenerife.

A detailed investigation of microgeographic genetic variation across the island of Tenerife was undertaken, with tissue samples being collected from 63 localities across the island, from all three precursor islands and intervening localities. Of the three mitochondrial genes sequenced for the earlier between-island study, cytochrome *b* had exhibited the finest level of resolution, so a 406 b.p. PCR fragment of the cytochrome *b* gene was subjected to a combination of denaturing gradient gel electrophoresis (DGGE) and direct sequencing. A total of 19 haplotypes were found, of which 16 were rare haplotypes found at only one or two localities. The three most common haplotypes showed a clear geographical distribution across the island (figure 3*a*). A pairwise distance matrix was generated, incorporating within-locality variation, and used to produce a Fitch–Margoliash tree. The tree shows that there are two main lineages, the first comprising haplotype A and the second haplotypes B and B′ and rare variants. The patristic distances were then input into a Mantel test as the dependent variable, with several independent variables derived from historical hypotheses describing different patterns of secondary contact between possible precursor populations from (i) Anaga and Adeje, (ii) Anaga and Teno, (iii) Anaga and joint Teno–Adeje, (iv) Teno and Adeje and (v) all three precursor islands separately (figure 3*b*). Also considered were (vi) a vicariance hypothesis (Pasteur & Salvidio 1985), which postulates that a ring of cloud around the mountain has resulted in two allopatric populations as population densities would be expected to be extremely low at the affected altitudes, and (vii) geographic proximity, the opportunity for gene flow being greater between geographically closer populations. The results showed that all three Anaga versus western-island hypotheses (i–iii), as well as the hypothesis of geographic proximity, could not be rejected. To eliminate the effects of intercorrelation of hypotheses, a partial Mantel test was then performed on these hypotheses, with the result that the Anaga versus Teno and Adeje hypothesis (hypothesis (iii)) alone could not be rejected. The timing of the split between the two lineages (just under 1 Ma ago) is consistent with the geological evidence, which indicates that Anaga may not have been linked with the rest of the

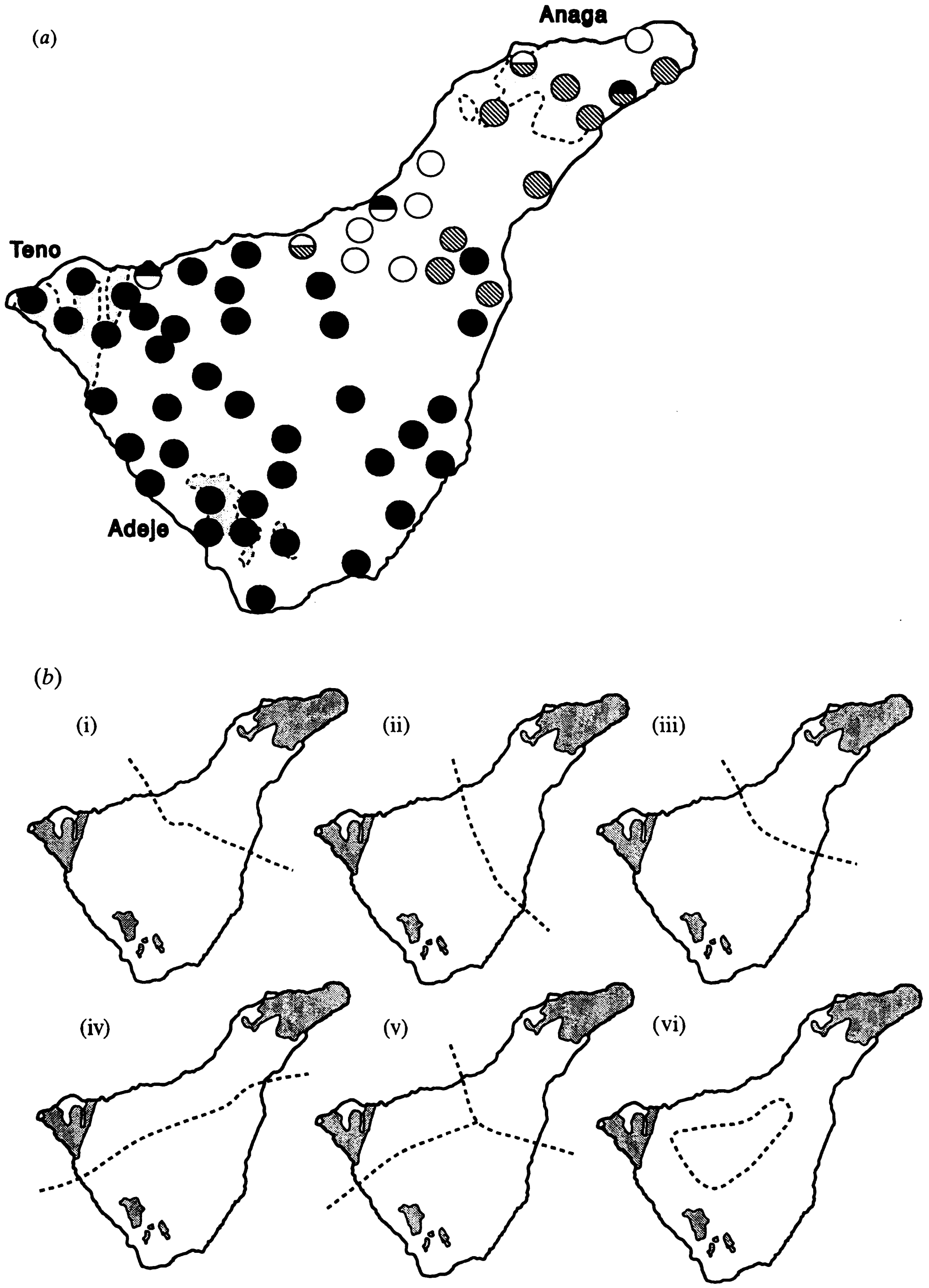

Figure 3. (*a*) The island of Tenerife, showing haplotype distribution across the island. Haplotype A is indicated by filled dots, haplotype B by empty dots, and haplotype B′ by hatched dots. Where more than one haplotype is present at any locality, the predominant haplotype is indicated. If haplotypes are present in equal proportions, they are indicated as appropriate. The positions of older rocks corresponding to the precursor islands are indicated by dashed lines. (*b*) The expected patterns produced by the various historical hypotheses. Dashed lines separate localities into categories. (i)–(v), Phylogenetic hypotheses based on dispersion and secondary contact from precursor islands, described in the text. Localities were assigned to categories on the basis of which ancient basaltic rock they were closest to. Hypotheses (i), (ii) and (iii) produce very similar patterns, with only one or two centrally positioned localities distinguishing them. (vi), Vicariance due to the mid-altitude cloud layer. (vii), An ecotone between lush forests on north-facing slopes and arid vegetation of the rest of the island.

island until later in the eruptive phase, possibly as late as 100000 years ago.

We can now use the data on the distribution of the phylogenetic lineages within the island to extract the phylogenetic component from morphological data, to see if lineage has an effect on the association found previously. This can be done in two ways. First, phylogeny (represented as a matrix of patristic distances among localities) is included as an independent variable together with matrices representing habitat type and geographic proximity. The colour pattern matrix (dependent variable) is then compared with these three independent matrices by using a partial regression in a simultaneous Mantel test.

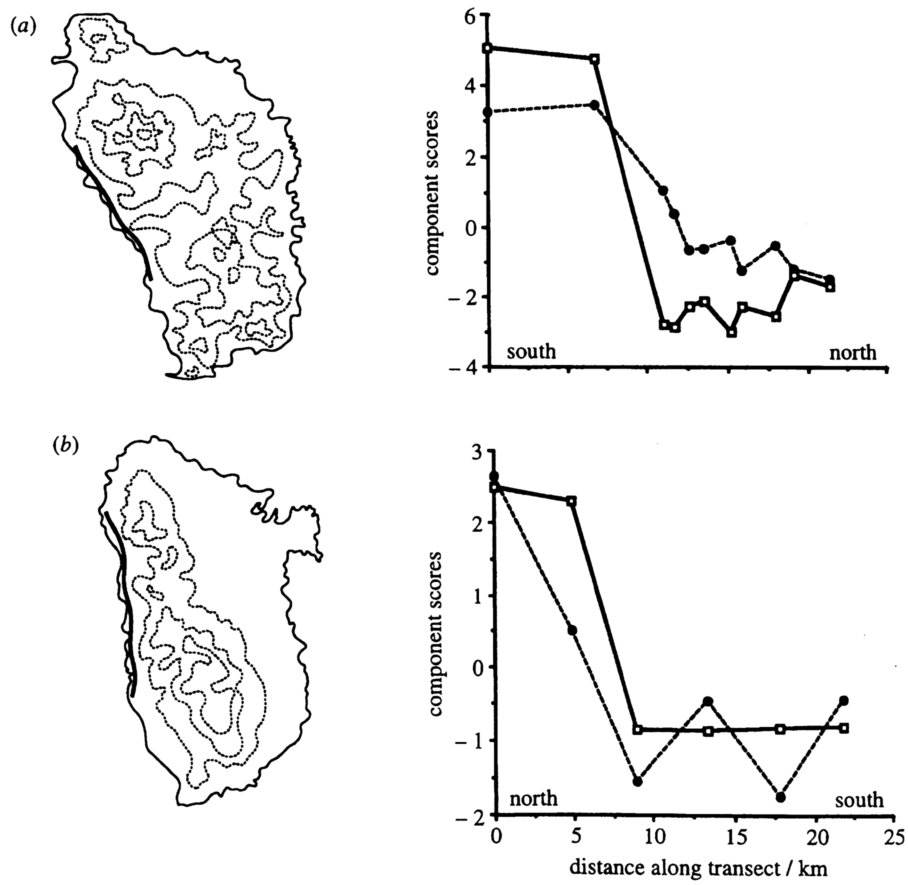

Figure 4. The Lesser Antillean islands of (*a*) Dominica and (*b*) Guadeloupe, with contours at 300 m intervals indicated by the dotted lines. The positions of the transect are marked by a heavy line. The adjacent graphs show the variation along the transect in the cytochrome *b* sequence (solid line) and scalation characters (broken line) in *Anolis oculatus* and *Anolis marmoratus* respectively. Note that the transects are plotted in opposite orientations because the ecological clines are in opposite directions in each island.

Secondly, tests for association of morphology and ecology can be carried out within each lineage separately (localities allocated according to which of the two main lineages predominates). When this is done with six colour pattern characters of mature males (Thorpe & Brown 1989), we find that there is a lineage effect, because lineage is significantly correlated with colour pattern in the former test. However, regardless of method of extracting lineage, the hypothesis that colour pattern variation in *G. galloti* reflects natural selection for habitat type cannot be rejected.

(*b*) **Anolis oculatus** *in Dominica* (*West Indies*)

Like Tenerife, Dominica is a volcanic island, with the combination of a high central mountain barrier and a constant wind direction resulting in the presence of a wide variety of habitats, from dry scrub to rainforest (Malhotra & Thorpe 1991*a*). The lizard (*Anolis oculatus*), a small semi-arboreal insectivore, is endemic to the island and displays a quite remarkable degree of geographic variation in colour pattern, body shape and size and scalation (Malhotra 1992; Malhotra & Thorpe 1991*a*), although these are not necessarily congruent. Hypothesis testing has shown this to be highly associated with ecological variation (Malhotra & Thorpe 1991*a*), and further evidence for natural selection was provided by manipulative field experiments involving the translocation of populations between habitats (Malhotra & Thorpe 1991*b*).

At the same time, we looked at patterns of variation in a closely related species, *Anolis marmoratus*, from the island immediately to the north of Dominica, Basse Terre, which is part of the Guadeloupean archipelago. This is an equally variable species, and we showed that many morphological characters showed parallel variation in relation to the same ecological factors as in *Anolis oculatus* (Malhotra & Thorpe 1994). These parallels can be interpreted in terms of parallel selection regimes because the islands have been independent since their origin and hence a common historical explanation is extremely unlikely.

However, there is a marked and relatively congruent cline in morphological characters of *A. oculatus* (body proportions, scalation and colour pattern) along the Caribbean coast of Dominica that does not fit this pattern, appearing out of proportion to the relatively subtle ecological clines along this cline compared with the rest of the island. To investigate the possibility of secondary contact zones (although no evident explanation for it exists), we sequenced 267 b.p. of the cytochrome *b* gene of mtDNA along a 22 km transect covering the area of the cline. This revealed the presence of a high level of genetic divergence (overall 12 %, with a maximum corrected pairwise difference of

9%) between northern and southern populations (Malhotra & Thorpe 1994). The geographic pattern of sequence divergence (figure 4) is congruent with morphological and environmental (moisture) gradients.

Because similar clines in morphology had also been observed in *A. marmoratus* along the Caribbean coast of Basse Terre, we sequenced cytochrome *b* for a similar transect. Unexpectedly, a similar pattern of cytochrome *b* divergence was found, which was also correlated with at least some morphological clines and with moisture gradients. It is extremely unlikely that similar phylogenetic events (e.g. multiple colonizations, vicariance resulting from volcanic activity) have taken place on both islands, with their positions bearing a similar relationship to environmental gradients (Brown *et al.* 1991). Another unusual feature is the high level of differentiation found. Among the 24 sequences from 11 populations of *A. oculatus*, there are 21 different haplotypes, with only 2 being shared between pairs of neighbouring populations. Similarly, in *A. marmoratus* there are 12 different haplotypes from 6 populations, with only 3 being shared between neighbours. If we use a commonly quoted rate for cytochrome *b* evolution (2.5% Ma^{-1}, which we used in the Canary island study without contradiction from other available evidence), then the time of divergence of the southern and northern lineages in *oculatus* would be of the order of 4 Ma. The volcanic activity associated with the origin of Dominica is thought to have begun in the late Pliocene (less than 4 Ma ago), with major, explosive eruptions associated with the appearance of most of the main volcanic cones in existence today continuing to as late as 100000 years ago (Martin-Kaye 1969). The maintenance of this degree of genetic isolation between populations separated by a distance of only a few kilometres without any major barriers to dispersal is also difficult to explain.

More information on divergence in other parts of the mitochondrial genome as well as that of nuclear markers may help to find an explanation for these patterns, and is the subject of continuing research. However, it is possible that some of the assumptions involved in a phylogenetic approach may be violated. The possibility of the operation of selection on the mitochondrial genome cannot be rejected at this stage, especially in the light of recent papers that have questioned the neutrality of cytochrome *b* evolution (Ballard & Kreitman 1994).

5. CONCLUSIONS

Observed geographic patterns may reflect the distribution of phylogenetic lineages, or adaptation to spatially varying environmental conditions. Molecular phylogenies can be useful in helping us to understand the underlying processes in a number of ways. Although the interpretation of mitochondrial genealogies in terms of the history of species should be treated with caution, they can nevertheless be seen to be useful at the intraspecific level, especially when combined with quantitative tests, such as Mantel (matrix correlation) tests.

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